

**THE POTENTIAL OF DIETARY LIPASES TO IMPROVE FAT
UTILIZATION IN YOUNG BIRDS**

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ABSTRACT

In comparison to the adult birds, young chickens and turkeys have a reduced ability to use dietary fat. Although this trend is true for all fats, it is particularly acute for fats containing a high proportion of long chain saturated fatty acids. The reason for the poor assimilation of fat has not been defined but has been speculated to be due to a deficiency of bile salts and/or pancreatic lipase. Lipase activity is low in the digestive tract of young birds but little research has been completed to examine the role of this enzyme in low fat utilization. Therefore it was of interest to investigate the role of lipase in fat malabsorption in young birds. It was hypothesized that fat malabsorption in young chickens and turkeys is due to an insufficiency of pancreatic lipase. It was further hypothesized that fat utilization in young birds can be improved by the use of dietary lipase sources.

For an exogenous lipase to be effective as a supplement to poultry diets and breakdown in the digestive tract, it must be able to withstand the conditions of the glandular stomach and the small intestine. The first *in vitro* experiment characterized the activity and stability of mammalian (crude and lyophilized porcine), fungal (*Rhizopus arrhizus* and *Aspergillus niger*) and bacterial (*Pseudomonas sp.* and *Chromobacterium viscosum*) lipase sources when exposed to conditions associated with the proventriculus of young birds (acidic pH, pepsin concentrations and internal body temperature). The results of this study demonstrated that bacterial *Pseudomonas sp.* and *Chromobacterium viscosum* lipase sources are more stable under conditions that mimic the glandular stomach of young

birds. *Aspergillus niger* lipase also showed a relatively high activity in acidic conditions. Mammalian crude porcine lipase was irreversibly inhibited in these conditions.

The second *in vitro* study tested crude porcine, bacterial *Pseudomonas sp.* and *Chromobacterium viscosum*, and fungal *Aspergillus niger* lipase sources under conditions which approximate the small intestine. The lipases were exposed to varying levels of trypsin, chymotrypsin and bile salts. Lipases which were inhibited by bile salts, were subsequently tested in the presence of porcine colipase. Under the conditions of this study, the *Pseudomonas sp.* lipase was more stable than the other lipases tested and was not inhibited by bile salts. It was concluded that *Pseudomonas sp.* lipase had potential as a supplement in poultry diets.

Two *in vivo* experiments were completed to test the use of *Pseudomonas sp.* lipase in diets of young broiler chickens and turkeys. The first experiment studied the impact of five levels of enzyme addition (0, 50, 100, 200 and 400 units per gram of diet) in a corn-soybean meal based diet containing 8% tallow and fed to broiler chickens from 0 to 35 days of age. Data collection included production parameters, fat digestibility, as well as other digestive tract characteristics. The results indicated that lipase addition has a negative impact on body weight gain, feed efficiency and fat digestibility. In addition, intestinal lipase activity actually decreased with higher levels of dietary lipase inclusion at 21 and 35 days of age. Analysis of feeds containing 0 and 400 units per gram of diet revealed that triglycerides had been nearly totally hydrolyzed to free fatty acids and glycerol during feed mixing and/or

storage in the enzyme supplemented treatment. It was suggested that the presence of free fatty acids would reduce the utilization of fat in the diet and may also have had a negative feed back on the pancreatic output of digestive enzymes. It was also concluded that the levels of lipase addition were too high. The second experiment utilized the same enzyme source at four lower levels (0, 20, 40 and 60 units per gram of diet) in diets for turkey poult under study from 0 to 21 days of age. The production results of this trial were similar but less severe than those seen for chickens.

In both the broiler chicken and turkey trials, the apparent digestibility of C18:0 was improved by the addition of lipase while that of C18:1 was decreased. The former may indicate insufficient endogenous lipase and the latter that the non-specific nature of the *Pseudomonas sp.* lipase hydrolyzes the fatty acids in the 2 position (predominate position of C18:1 in tallow) of the glycerol molecule thereby reducing their digestibility.

The results of this research do not permit the acceptance or rejection of thesis hypotheses. The unexpected finding that the lipase used in *in vivo* experiments can hydrolyze triglycerides prior to consumption by birds is undesirable and raises a number of questions. It is undesirable because of the increased potential for auto-oxidation of free fatty acids in comparison to triglycerides. The non-specific nature of the *Pseudomonas sp.* and complete hydrolysis of triglycerides is also inappropriate because monoglycerides enhance micellar formation and improve absorption of free fatty acids. The high free fatty acid content of the diets may also

play a role in feed back inhibition of pancreatic enzyme secretion. Therefore, these undesirable effects may have hidden the benefit of a dietary lipase source. Questions are also raised as to whether the form of enzyme application (liquid or dry) has an impact on lipid hydrolysis and what impact enzyme dosage has on the success of lipase supplementation. Further research is required to identify a lipase source and form that is active in the digestive tract and not the feed, is glycerol site specific and produces monoglycerides as well as free fatty acids, and is resistant to the conditions of the gastric stomach and small intestine.

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TABLE OF CONTENTS

	Page
PERMISSION TO USE.....	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	vi
LIST OF TABLES.....	xiii
LIST OF FIGURES.....	xv
LIST OF APPENDICES.....	xvii
 CHAPTER 1	
INTRODUCTION.....	1
1.1 Background Information.....	1
2.1 Hypothesis.....	3
 CHAPTER 2	
LITERATURE SURVEY.....	4
2.1 Use of Fat and Oils in Poultry Diets.....	4
2.2 Fat Digestion and Absorption.....	4
2.3 Factors Affecting Digestion and Absorption.....	7
2.3.1 Fatty acid saturation and chain length.....	7

2.3.1.1 Micelle formation.....	7
2.3.1.2 Lipase activity.....	8
2.3.2 Fatty acid position on the glycerol molecule.....	10
2.3.3 Fatty acid binding protein and fat utilization.....	11
2.3.4 Lipid melting point.....	11
2.3.5 Nutrient levels.....	12
2.3.5.1 Level and type of fat intake.....	12
2.3.5.2 Protein level.....	13
2.3.5.3 Mineral level.....	14
2.3.6 Inhibitors and antinutritional factors in the diet.....	15
2.3.7 Aflatoxin and lipid absorption.....	18
2.3.8 Viscosity and lipid digestion and absorption.....	19
2.4 Digestion and Absorption of Lipids in Very Young Birds.....	20
2.4.1 Development of lipid digestion with age.....	20
2.4.1.1 Fat digestion before hatching.....	20
2.4.1.2 Fat digestion after hatching.....	21
2.4.1.2.1 Effect of rate of feed passage.....	21
2.4.1.2.2 Bile salt status.....	22
2.4.1.2.3 Digestive enzyme status.....	23
2.5 Dietary Supplements.....	25
2.5.1 Dietary bile salt supplements.....	25
2.5.2 Lipase supplements.....	27

CHAPTER 3

STABILITY OF PORCINE AND MICROBIAL LIPASES TO CONDITIONS WHICH APPROXIMATE THE PROVENTRICULUS OF YOUNG BIRDS.....

46

3.1 Abstract.....	46
3.2 Introduction.....	47
3.3 Materials and Methods.....	50
3.3.1 Measurements of lipase activity.....	50
3.4 Results.....	51
3.4.1 Initial rate of tributyrin hydrolysis.....	51
3.4.2 Kinetics of <i>Pseudomonas</i> and crude porcine lipase at pH 7 and 8.....	53
3.4.3 Effect of pH on lipase activity.....	53
3.4.4 Effect of pre-incubation conditions on lipase activity.....	56
3.5 Discussion.....	56
3.6 Conclusions.....	60

CHAPTER 4

STABILITY OF PORCINE AND MICROBIAL LIPASES TO CONDITIONS WHICH APPROXIMATE THE SMALL INTESTINE OF YOUNG BIRDS.....

62

4.1 Abstract.....	62
-------------------	----

4.2 Introduction.....	63
4.3 Materials and Methods.....	65
4.3.1 Measurement of lipase activity.....	66
4.3.2 Statistical analysis.....	67
4.4 Results.....	67
4.5 Discussion.....	73
4.6 Conclusions.....	77

CHAPTER 5

THE EFFECT OF DIETARY SUPPLEMENTATION OF <i>PSEUDOMONAS SP.</i> LIPASE ON FAT DIGESTIBILITY IN BROILER CHICKENS.....	78
5.1 Abstract.....	78
5.2 Introduction.....	79
5.3 Materials and Methods.....	82
5.4 Results.....	86
5.5 Discussion.....	97
5.6 Conclusions.....	101

CHAPTER 6

THE EFFECT OF DIETARY SUPPLEMENTATION OF <i>PSEUDOMONAS SP.</i> LIPASE ON FAT DIGESTIBILITY IN TURKEY POULTS.....	102
----------------------------------------------------------------------------------------------------------------------------------	------------

6.1 Abstract.....	102
6.2 Introduction.....	103
6.3 Materials and Methods.....	105
6.4 Results.....	108
6.5 Discussion.....	115
6.6 Conclusions.....	117

CHAPTER 7

GENERAL SUMMARY AND CONCLUSIONS.....	118
---------------------------------------------	------------

CHAPTER 8

REFERENCES.....	122
APPENDICES.....	151

LIST OF TABLES

	Page
2.1 Beneficial effects of fats and oils in poultry diets.....	5
2.2 Substrate specificity of lipases sources.....	38
4.1 The effects of sodium taurodeoxycholate on the activity of lipases at 23 and 40 C.....	72
5.1 Composition and nutrient content of basal diets.....	83
5.2 The effect of dietary <i>Pseudomonas sp.</i> lipase on body weight gain (g) in broiler chickens.....	87
5.3 The effect of dietary <i>Pseudomonas sp.</i> lipase on feed intake in broiler chickens.....	88
5.4 The effect of dietary <i>Pseudomonas sp.</i> lipase on feed to gain ratio in broiler chickens.....	89
5.5 The effect of dietary <i>Pseudomonas sp.</i> lipase on actual and proportional pancreas in broiler chickens.....	90
5.6 The effect of dietary <i>Pseudomonas sp.</i> on the lipase activity of pooled duodenal and jejunal contents in broiler chickens.....	91
5.7 The effect of dietary <i>Pseudomonas sp.</i> lipase on the apparent fat digestibility in broiler chickens.....	93
5.8 Fatty acid composition of tallow and basal diets fed to broiler chickens (%).....	94
5.9 The effect of dietary <i>Pseudomonas sp.</i> lipase on the digestibility of individual fatty acids in broilers (%).....	95
5.10 The amount of tri, di and monoglycerides and free fatty acids in tallow and diets containing 8% tallow with or without dietary <i>Pseudomonas sp.</i> lipase (%).....	96
6.1 Composition and nutrient content of basal diets.....	106

6.2	The effect of dietary <i>Pseudomonas sp.</i> lipase on body weight gain in turkey poults.....	109
6.3	The effect of dietary <i>Pseudomonas sp.</i> lipase on feed intake in turkey poults.....	110
6.4	The effect of dietary <i>Pseudomonas sp.</i> lipase on feed to gain ratio in turkey poults.....	111
6.5	The effect of dietary <i>Pseudomonas sp.</i> lipase on the apparent fat digestibility in turkey poults.....	112
6.6	Fatty acid composition of tallow and basal diet fed to turkey poults...	113
6.7	The effect of dietary <i>Pseudomonas sp.</i> lipase on digestibility of individual fatty acids in turkey poults.....	114

LIST OF FIGURES

	<u>Page</u>
2.1 Development of digestive enzymes in pancreatic tissue and intestinal contents of poult with age.....	24
2.2 Trypsin and bilirubin outputs during continuous intraduodenal perfusion of EAAs before, during and after addition of sodium taurocholate in human subjects.....	31
2.3 Schematic diagram illustrated the relation between the ratio of fatty acids to bile acids (FA:BA) in the intestine on CCK release, FA absorption and gallbladder contraction.....	31
3.1 Activity of <i>Pseudomonas sp.</i> lipase (PL1) at 40 C and ~ 50 units lipase.....	52
3.2 Activity of <i>Pseudomonas sp.</i> lipase (PL1) and crude porcine lipase (CPL) at pH 7 (□) and pH 8 (●) and different tributyrin concentrations.....	54
3.3 Activity of mammalian (A), fungal (B) and bacterial (C) lipases at varying pHs, 40 C and 2 ml tributyrin.....	55
3.4 Activity of mammalian (top), fungal (middle) and bacterial (bottom) lipases under varying conditions, pH 7, 40 C and 2 ml tributyrin.....	57
4.1 The effect of pre-incubation at 40 C for 30 minutes with and without trypsin on the activity of <i>Aspergillus niger</i> lipase (A), <i>Chromobacterium viscosum</i> lipase (B), <i>Pseudomonas sp.</i> PL1 (C) and <i>Pseudomonas sp.</i> PL2 (D) lipase sources at pH 7 and 40 C using tributyrin as substrate.....	68
4.2 The effect of chymotrypsin on lipase activity after various pre-incubation conditions. Activity of <i>Aspergillus niger</i> lipase (A), <i>Chromobacterium viscosum</i> lipase (B), <i>Pseudomonas sp.</i> PL1 (C)	

	and <i>Pseudomonas sp.</i> PL2 (D) lipase sources at pH 7, and 40 C using tributyrin as the substrate.....	69
4.3	The effect of varying concentrations of a 1:1 mixture of Na-cholate:Na-deoxycholate (NaCD) on the activity of <i>Aspergillus niger</i> (A), <i>Chromobacterium viscosum</i> (B), <i>Pseudomonas sp.</i> PL1 (C) and <i>Pseudomonas sp.</i> PL2 (D) lipase sources measured at pH 7 and 40 C using tributyrin as the substrate.....	71
4.4	The effects of Na taurodeoxycholate on the activity of crude porcine lipase (CPL), <i>Chromobacterium viscosum</i> lipase (CVL) and <i>Aspergillus niger</i> lipase (ANL) at pH 7, 23 (open symbols) or 40 C (solid symbols) , with (●, ○) or without (■, □) the addition of porcine colipase (PCL).....	74

LIST OF APPENDICES

	Page
I Peak retention times of standard fatty acids.....	151
II Peak retention times of dietary fatty acids.....	152
III Peak retention times of feces fatty acids	153
IV Tri, di, monoglycerides and free fatty acids of pure beef tallow.....	154
V Broiler diet containing 8% tallow and 400 u/g of diet <i>Pseudomonas</i> <i>sp.</i> lipase.....	155
VI Broiler diet containing 8% tallow and no added <i>Pseudomonas sp.</i> lipase.....	156

1.0 INTRODUCTION

1.1 Background Information

The largest proportion of the cost of a balanced poultry diet is energy. In modern poultry feed formulation, where demand for using linear programming techniques is increased, the exact knowledge of the available energy content is very important because the inclusion or rejection of a particular feedstuff in the least cost ration is greatly dependent on the specified energy values.

Fats are important ingredients of poultry diets. The incorporation of fats into the diets has received an increasing amount of attention in recent years. Fats are the most potent feed energy source and provide many other beneficial effects in the poultry diets. Based on energy content and price compared to other energy sources, it is often economical to add fat to poultry diets.

Among fats, those characterized by a high proportion of saturated fatty acids are not well utilized in young birds. This problem diminishes with increasing age. Many physiological functions related to digestion and absorption of fats are not mature at hatching and continue to develop for several weeks. Several aspects of the process of digestion and absorption of fats have been speculated to be deficient in young birds but the exact reason for fat malabsorption is still lacking. However, the lack of maturity in pancreatic secretions, in particular lipase, may be an important factor causing low utilization of saturated fatty acids in very young chickens.

In recent years, the major improvements in nutrition have come from the use of new feedstuffs and additives, and among the latter, enzymes have been of most interest. Although some exogenous enzymes have been used for improving feed efficiency in poultry diets, most of them have been for hydrolysis of non starch polysaccharide (NSP) fractions in cereal grains or grain cell walls. Hydrolysis of NSP can indirectly improve fat digestion and absorption, but information concerning how dietary lipase might directly increase the utilization of dietary lipid in poultry is very rare.

Interest in the industrial application of lipases has increased markedly in the last decade due to potential uses in industry and medicine. Examples from the food industry include the development of cheese flavour and ripening, the improvement of aroma and acceleration of fermentation of apple wine, and the improvement of the whipping properties of egg white. The use of lipases in medicine for treatment of steatorrhoea in human pancreatic insufficiency is also very important. Use of microbial lipases in the detergent industry is another example of the industrial application of lipases. The interest in lipase application is likely to increase their availability and lower prices. As a consequence this may allow lipase use in animal, and in particular poultry feeding programs.

1.2 Hypothesis

It is hypothesized that the main factor responsible for the poor utilization of saturated fatty acid is lipase insufficiency in young birds. The use of dietary lipase, especially microbial sources, is virtually non-existent in the poultry industry and research is required to determine whether this technique has practical value. This study was designed to investigate the ability of microbial and mammalian lipase sources to survive and be active in conditions which mimic the digestive tract of birds and to test promising sources in young birds fed a diet containing a high level of saturated fatty acids.

2.0 LITERATURE SURVEY

2.1 Use of Fats and Oils in Poultry Diets

Fats and oils are the most potent feed energy source. As a consequence, dietary fat allows for greater flexibility in feed formulation, and reduces the total weight and volume of feed that must be mixed, handled and transported (Ensminger *et al.*, 1990). Some of the beneficial effects of fats and oils are presented in Table 2.1. Therefore based on energy content and price compared to grain and grain by-products, it is often economical to use fats and oils in poultry diets.

2.2 Fat Digestion and Absorption

In monogastric species, fats are digested and absorbed primarily in the jejunum, but considerable absorption can take place in the ileum (Renner, 1965; Hurwitz *et al.*, 1973). The digestion of fat occurs as a result of the action of bile which emulsifies the fat, thus greatly increasing the surface area, and pancreatic lipase, an enzyme which hydrolyses fatty acids from the glycerol molecules of triglycerides (Scott *et al.*, 1982; Ensminger *et al.*, 1990). Hydrolysis of emulsified triglyceride by pancreatic lipase can be inhibited by bile salts that displace lipase from the interface and make it inactive. This inhibition can be reversed by another pancreatic protein, colipase (Borgstrom, 1975; Erlanson-Albertson, 1992a). Colipase acts as a specific co-factor of pancreatic lipase that enables the latter

TABLE 2.1 Beneficial effects of fats and oils in poultry diets

Action	Reference
Increase the caloric density of the ration	Ensminger <i>et al.</i> (1990)
Control dustiness	Ensminger <i>et al.</i> (1990)
Reduce wear on mixing equipment	Ensminger <i>et al.</i> (1990)
Provide a protective agent for some micro nutrients	Ensminger <i>et al.</i> (1990)
Increase palatability of the feed	Ensminger <i>et al.</i> (1990)
Prevent particle separation	Ensminger <i>et al.</i> (1990)
Act as a carrier of fat soluble vitamins and pigments	Ensminger <i>et al.</i> (1990)
Act as a source of essential fatty acids	Ensminger <i>et al.</i> (1990)
Decrease the specific dynamic effect of feed	Sell <i>et al.</i> (1979; Fuller (1980)
Act as a growth promotion factor	Fuller and Renden (1979); Sell <i>et al.</i> (1986)

enzyme to adsorb at the oil-water interface (Sternby and Borgstrom *et al.*, 1979). Colipase itself has no enzymatic activity (Erlanson-Albertson, 1992b) but is necessary for hydrolysis of dietary fats by pancreatic lipase in the intestinal lumen (Borgstrom *et al.*, 1979a; Rinderknecht, 1986; Erlanson-Albertson, 1992a, b). Colipase in the pancreas and pancreatic juice is a precursor form that is converted to its physiologically active form by trypsin (Borgstrom *et al.*, 1979b; Rinderknecht, 1986). The biological lipase protein co-factor in chickens is similar to colipase from mammalian species (Canioni *et al.*, 1975; Bosc-Bierne *et al.*, 1984; Erlanson-Albertson, 1992a). Colipase binds to lipase in a 1:1 molar complex and also binds to the bile salt covered triglyceride (Erlanson-Albertson, 1992b). This ratio is needed for optimal activation of lipase by colipase (Erlanson-Albertson, 1992a).

When fat emulsified by bile salts, comes into contact with the lipase and colipase in the duodenum, it is broken down into monoglycerides and fatty acids. Short-chain fatty acids can be absorbed directly into the enterocyte of the small intestine and transported to the portal circulation. Monoglycerides and insoluble fatty acids are emulsified by conjugated bile salts to form micelles. After attaching to the surface of epithelial cells, the micelles enable these compounds to be absorbed into the mucosal cell. Inside the cell, the long-chain fatty acids and monoglycerides are re-esterified forming triglycerides. Triglycerides then combine with cholesterol, lipoproteins and phospholipids forming chylomicrons.

Chylomicrons are transported from intestinal enterocytes to the systemic circulation of the body. In turn, they are transported to various tissues, particularly the liver, where they are used in synthesis of various compounds required by the body, metabolized as a source of energy or stored in the tissues as fat deposits (Scott *et al.*, 1982; Ensminger *et al.*, 1990).

2.3 Factors Affecting Digestion and Absorption of Lipids

Effective digestion and hydrolysis of dietary triglycerides requires the coordination of lipase, colipase, bile salts and phospholipids together with a sufficient degree of intestinal motility. Limited availability of one or more of these factors may reduce utilization of dietary lipids (Escribano *et al.*, 1988; Krogdahl and Sell, 1989). Fat absorption can also be influenced by other factors, some of which are described in the following sections.

2.3.1 Fatty acid saturation and chain length

2.3.1.1 Micelle formation

Fatty acid saturation and chain length influence fat digestion and absorption. Long chain saturated fatty acids (LCSFAs) are poorly utilized by poultry in comparison to short or medium length saturated fatty acids and unsaturated fatty acids. Long chain poly-unsaturated fatty acids (polar solutes) are more efficiently digested and absorbed than long chain saturated fatty acids (non-

polar solutes) in broiler chicks because of their increased solubility in bile salt solutions and their higher micellar formation capacity (Freeman *et al.*, 1968; Wiseman *et al.*, 1986). The presence of monoglycerides or unsaturated fatty acids positively influence saturated fatty acid utilization because of the increased potential for micellar formation. Long chain saturated fatty acids in free form are absorbed and utilized less than when present in the ester form (Bayley and Lewis, 1965). A high proportion of free fatty acids in broiler diets decreases the apparent retention of dietary fat (Wiseman and Salvador, 1991).

Short and medium chain fatty acids (less than 14 carbons) are generally saturated but readily form micelles. Therefore the digestibility of triglycerides containing a high proportion of medium chain fatty acids is high regardless of the dietary ratio of unsaturated to saturated fatty acids (Hamilton and McDonald, 1969).

2.3.1.2 Lipase activity

Lipase (EC 3.1.1.3) is necessary for triglyceride hydrolysis. It acts at the oil-water interface (Erlanson-Albertson, 1992a, b). Bile salts can inactivate lipase at the interface but colipase can overcome the presence of excess bile salts. *In vitro* studies have shown that in addition to a lipid-water interface and colipase, some other factors are required for the efficient functioning of lipase. It was found that in the presence of colipase, free fatty acids differentially affect lipase activity

(Larsson and Erlanson-Albertson, 1981, 1986). Oleic (C18:1) and linoleic (C18:2) acids greatly increased lipase activity, while lauric acid (C12:0) only moderately facilitated lipase activity. Saturated free fatty acids with less than 10 carbons had little effect on lipase activity, and the LCSFA stearic acid (C18:0) inhibited lipase activity. Larsson and Erlanson-Albertson (1981, 1986) suggested that the reactivation of the lipase-colipase complex by certain fatty acids may be related to an increase in lipase specific activity by increasing binding between colipase and lipase or to a change in the quality of the lipid-water interface.

An *in vitro* study by Kuiken and Behnke (1994) demonstrated the role of certain fatty acids on lipase activity in the absence of colipase. They showed that porcine pancreatic lipase specific activity increased about 15 times in the presence of 1 mM oleic acid. The ability of oleate to increase lipase activity did not require the presence of colipase. Most *cis*-unsaturated fatty acids (18 and more carbons) increased lipase activity by 14 to 15 fold. Stearic acid did not increase lipase specific activity while medium chain saturated fatty acids capric acid (C10:0) and lauric acid increased lipase specific activity about 3 times.

The position of the first double bond in unsaturated fatty acids affects its ability to increase lipase activity (Kuiken and Behnke, 1994). If the first double bond of the fatty acid is at carbon 6, 9 or 11, then the fatty acid effectively increases lipase activity but if it is at the 13th carbon, the fatty acid is less than half as effective.

The geometry of double bond is also an important determinant for lipase activity. Kuiken and Behnke (1994) showed that elaidic acid (C18:1, *trans*-9) doubles lipase specific activity whereas oleic acid (C18:1, *cis*-9) caused 7 times more lipase specific activity than elaidic acid.

These *in vitro* results clearly suggest the role of free fatty acids and their saturation, chain length as well as the geometry of double bonds in lipase activity. Further research is required to clarify their effects in *in vivo* studies.

2.3.2 Fatty acid position on the glycerol molecule

The position of saturated fatty acids on the glycerol molecule may influence their digestibility, due to the fact that pancreatic lipase selectively hydrolyses the bond at the 1 and 3 positions of the glycerol moiety (Scott *et al.*, 1982). Therefore, fats such as lard, which have palmitic acid predominantly esterified at the 2 position, may be digested slightly more efficiently (2-4%) because of the greater micellar formation potential of a monoglyceride than that of the free acid hydrolyzed from the 1 and 3 positions (Freeman *et al.*, 1968). Unsaturated fatty acids occupy the 2 position of glycerol in most animal fats and in tallow most of the stearic and palmitic acids are found in the 1 and 3 positions (Scott *et al.*, 1982). In most oils, saturated fatty acids are also found in positions 1 and 3 and linoleic acid predominates in the 2 position.

2.3.3 Fatty acid binding protein and fat utilization

The intestinal mucosa of the chicken contains a fatty acid binding protein (FABP) that is suggested to be involved in the absorption of fatty acids across the enterocyte membrane (Katongle and March, 1979). The FABP may be a limiting factor in birds with poor utilization of dietary fat. The amount of FABP in chickens at hatching and during early life is low but increases with age (Katongle and March, 1980). In chickens the concentration of FABP is highest in the proximal portion of the intestine and decreases distally (Katongle and March, 1979). It has been suggested that the better utilization of unsaturated compared to saturated fatty acids is related to FABP. Poly-unsaturated fatty acids, compared to saturated fatty acids, have a higher affinity to FABP (Ockner and Manning, 1974, 1976). Short and medium chain fatty acids have no affinity to cytosolic FABP (Ockner and Manning, 1974; Bass, 1985).

2.3.4 Lipid melting point

The degree of fluidity of lipids and lipid containing structures in the animal are of major importance. Fluidity is usually judged by measuring the fatty acid content of fats or oils. The melting point of saturated fatty acids increases with increasing chain length (Wiseman, 1984). Saturated fatty acids with 12 or more carbon atoms are solid at body temperature. Fatty acids with a double bond have a lower melting point than saturated chain fatty acids of the same length. The

presence of each additional double bond decreases the lipid melting point. The melting point also depends upon the geometry of the double bonds. *Trans* fatty acids have higher melting points than *cis* fatty acids. The position of the double bond also changes this property. For example, oleic acid (C18:1, *cis* double bond at position 9) has a melting point of 13.4 C, elaidic acid (C18:1, *trans*-9 isomer) melts at 43.7 C, and vaccenic acid (18:1 *trans* isomer) but with a double bond at position 11 melts at 39.0 C (Wiseman, 1984). Since melting point is a characteristic of the fatty acid showing its chain length, saturation, number of double bonds and the geometry of double bonds in *cis* or *trans* form, it indirectly affects lipid digestion and absorption.

2.3.5 Nutrient levels

The apparent digestibility of fat is influenced by the dietary levels of fat, protein and minerals. The details of each of these influences are discussed in the following sections.

2.3.5.1 Level and type of fat intake

Dietary fat (level and type) affects fat digestion. The effect of 0 to 10% dietary rapeseed oil, beef fat and their combinations in turkey poults from 1 to 6 weeks of age was studied by Salmon (1977). Mean fat digestibility increased from 79.3 to 95.6% when 10% beef fat (high in saturated fatty acids) was replaced with

10% rapeseed oil (high in unsaturated fatty acids). Age had a definite effect on fat digestion only when beef fat was fed. Fat digestibility increased from 71.1 to 82.9% from 1 to 6 weeks of age. Increasing the level of dietary saturated fatty acids by increasing the amount of beef fat from 0 to 10% decreased digestibilities of palmitic and stearic fatty acids (89.8 vs 69.2 for C16:0; 91.9 vs 57.2 for C18:0). There was no effect of fat levels when more unsaturated fatty acids were fed by turkey poults.

It has been suggested that the lack of co-ordination between lipase and colipase secretion may cause lower fat digestibility when a high fat diet is fed by rats (Ouagued *et al.*, 1980). The authors suggested that when rats were fed a high fat diet, colipase was secreted into the small intestine faster than lipase at the beginning of active period of feeding. This in turn, caused the depletion of pancreatic colipase when the load of fat reached the small intestine. It is also suggested that the higher solubility and micellar formation capacity of the diets containing higher unsaturated fatty acids make them more efficient for digestion and absorption (Wiseman *et al.*, 1986).

2.3.5.2 Protein level

Relative amounts of dietary protein may influence fat digestion (Gidez, 1973). Feeding a high protein diet (40% casein) to rats caused a rapid increase in colipase secretion (Girard-Globa *et al.*, 1980). In addition to colipase level, all of

the hydrolases increased in parallel in the pancreas. This increase may be because of the sudden activation of hydrolases synthesis due to the influx of amino acids. Levels of colipase rose faster than other pancreatic secretions including lipase (Ouagued *et al.*, 1980). Colipase responded to protein intake even in low lipid diets (2% lard) by increasing synthesis 3 fold when the casein level of the diet was raised from 18 to 40%. This response is probably related to a higher protein intake. Ouagued *et al.* (1980) showed that the caloric intake from protein source was increased from 12.8 to 26.6 kcal/day when the casein level of the diet was increased from 18 to 40%.

2.3.5.3 Mineral Level

High levels of dietary minerals decrease fat digestion. There are numerous reports indicating that fat retention and diet ME decrease when poultry diets contain high levels of fat and calcium (Hakansson, 1974; Sibbald and Price, 1977; Atteh and Leeson, 1984, 1985). Atteh and Leeson (1985) showed that increasing the calcium level in broiler chicken diets increased the proportion of the digesta fat that was present as soap. They also reported that the proportion of digesta and excreta fat present as soaps is dependent on the type of fatty acid supplemented. They showed that the addition of 8% palmitic acid (C16:0) to broiler diets containing 0.8 or 1.2% calcium resulted in an increase in excreta soap, a decrease in fat retention and ME of the diets containing palmitic acid relative to those birds

fed the diet containing a control diet and no added free fatty acid. A mixture of 50/50 palmitic and oleic acid (C18:1) added to the control diet caused the same response as palmitic acid. However, the negative effects were not as severe as when only palmitic acid was added to the diet.

High levels of calcium and magnesium depress the digestibility of long chain fatty acids in rats (Cheng *et al.*, 1949). High levels of dietary calcium increase fecal output and total fecal lipids. In fact, the amount of calcium has more effect on fecal fat excretion than the amount of fat in the diet. Calcium binds with fatty acids in the small intestine and makes them unavailable for absorption (Drenick, 1961; Day *et al.*, 1975; Lupton *et al.*, 1994). Raising human calcium intake by 6 grams per day increased fecal excretion from 106 to 131 grams and fecal fatty acids from 7.9 to 16.8 mM (Saunders *et al.*, 1988).

Although the lower digestibility of LCSFAs can be caused by high levels of minerals in the diet, the LCSFAs also decrease the availability of minerals including calcium and magnesium. These minerals are important nutrients and their deficiencies decrease the performance of the birds.

2.3.6 Inhibitors and antinutritional factors in the diet

Lipid digestion and the lipase activity of digesta are decreased in young chicks fed a diet containing a high concentration of tannin-rich field bean hulls (Longstaff and McNab, 1991b). Low dietary tannins (20 and 50 g field-bean

hulls/kg diet) can increase the activity of lipase in the digestive tract of the chicks but higher concentrations (150 and 300 g hulls/kg diet) inhibit lipase activity in digesta (Longstaff and McNab, 1991a). *In vitro* studies have also shown that tannins decrease lipase activity (Griffiths, 1979; Horigome *et al.*, 1988). Mole and Waterman (1985) suggested that tannins cause conformational changes in the substrate which change the accessibility of lipase to the substrate.

Dietary fibre influences digestive enzymes. Schneeman (1978) reported that the availability of enzymes such as lipase, trypsin and chymotrypsin can be limited by their adsorption onto fibers such as xylan, cellulose, wheat bran and rice bran. The formation of enzyme-fiber complexes in the gut decrease the interaction of enzymes and substrates (Gagne and Acton, 1983).

Phytate and its hydrolysis products, myo-inositol phosphate esters present in whole grain and oil seeds, may decrease lipid digestion. This may be mediated by a reduction in lipase activity as indicated *in vitro* by Knuckles (1988). Pre-incubation of porcine lipase with 1 to 12 mM phytate or myo-inositol phosphate esters caused a linear reduction in lipase activity. The inhibition was positively correlated with the degree of phosphorylation. Addition of colipase didn't change the results.

The utilization of phytate can be influenced by dietary fat (Matyka *et al.*, 1990). Beef tallow in contrast to unsaturated fats decreases the utilization of phytate and phytin phosphorous. They suggested a decline in phytase activity in

the presence of saturated fatty acids in the diet may be responsible for the lower efficiency of phytate in broiler chickens.

Raw soybeans contain compounds that inhibit pancreatic lipase (Satouchi and Matsushita, 1976). These components are partially heat stable and decrease lipid digestion and absorption in rats (Khalifa *et al.*, 1994). Lipase inhibition by soybean proteins does not result from the formation of a soluble complex between enzyme and inhibitor (Gargouri *et al.*, 1984). Soybean inhibitors bind to the oil-water interface and prevent enzyme interaction with the substrate. Gargouri *et al.* (1984) showed that lipase sources respond differently to the soybean protein under *in vitro* conditions. *Rhizopus delemar* lipase and pancreatic lipase were strongly inhibited while activity of *Rhizopus arrhizus* lipase remained relatively unchanged. They suggested that the binding capacities of these lipases to protein-modified lipid substrate are different.

Wheat bran, wheat germ and wheat flour proteins can also inhibit pancreatic lipase activity (Borel *et al.*, 1989; Tani *et al.*, 1994). The inhibition of pancreatic lipase by the wheat germ proteins is related to their ability to interact with the emulsified substrate and to hinder the absorption of the enzyme on the interface (Cara *et al.*, 1992).

Tani *et al.* (1994) studied the effect of wheat proteins on the activity of *Rhizopus arrhizus*, *Chromobacterium viscosum*, porcine and *Candida cylindracea* lipases. Porcine lipase was fully inhibited, *Candida cylindracea* was partially

inhibited, while *Rhizopus arrhizus* and *Chromobacterium viscosum* lipase were not inhibited. They suggested that the proteins in wheat flour may play a role in fat digestion.

Since plant proteins are used routinely as a large part of dietary proteins, the interactions between protein inhibitors and enzymes in fat and other nutrient digestibilities must be considered important.

2.3.7 Aflatoxins and lipid absorption

The deleterious effect of aflatoxins on lipid absorption has been shown in many species including birds (Shank and Wogan, 1966; Donaldson *et al.*, 1972). Aflatoxin has many deleterious effects in chickens but poor feed efficiency is a very marked sign (Smith and Hamilton, 1970; Smith *et al.*, 1971; Osborne *et al.*, 1975). Aflatoxin produces a significant steatorrhoea in very young chicks and this effect is likely related to this mycotoxin's negative effect on pancreatic lipase secretion, even at very low levels (Osborne and Hamilton, 1981). The latter authors showed a significant enlargement of relative pancreas weight. They also showed that the bile concentration was decreased significantly by growth inhibitory levels of dietary aflatoxin.

2.3.8 Viscosity and lipid digestion and absorption

Viscous fiber polysaccharides are known to affect lipid digestion and absorption more than other dietary nutrients. A severe decrease in fat retention by viscous fiber polysaccharides has been reported with the largest effect on longer chain saturated fatty acids (Ward and Marquardt, 1983; Bedford *et al.*, 1996). Choct and Annison (1992) reported that the digestibility of unsaturated fatty acids C18:1, C18:2, C18:3, and C20:2 were not significantly affected by the addition of viscous soluble wheat pentosans, whereas those of all saturated fatty acids C14:0, C16:0, C18:0, and C20:0 were significantly decreased. It is also suggested that soluble viscous fibres can alter lipid assimilation by reducing emulsification of dietary lipids (Pasquier *et al.*, 1990). The mechanism by which viscosity decreases fat digestion and absorption is not totally understood. It is suggested that an increase in the viscosity of the intestinal contents decreases the rate of diffusion of substrates and digestive enzymes and hinders their interaction at the mucosal surface (Ikegami *et al.*, 1990). Viscous polysaccharides might directly complex with digestive enzymes and reduce their activity (Ikeda and Kusano, 1983). Soluble fibres can decrease lipase activity (Longstaff and McNab, 1991a; Almirall *et al.*, 1995). The inhibitory effect of soluble fibres on lipase is more than that noted for other digestive enzymes (Isaksson *et al.*, 1982). Viscosity induced proliferation of intestinal microorganisms may also result in deconjugation of bile salts which in turn reduces the effectiveness of the digestion process (Classen,

1996).

Bedford (1996), in a review on the effect of dietary microbial enzymes on digestion, concluded that these enzymes can improve digestion of nutrients including fat in birds by removing anti-nutritional effects of viscosity caused by NSP which interfere with the normal processes of digestion and absorption.

2.4 Digestion and Absorption of Lipids in Very Young Birds

2.4.1 Development of lipid digestion with age

Fat digestion and absorption in birds develops with age. Factors involved in this process are discussed in the following sections.

2.4.1.1 Fat digestion before hatching

Yolk lipids are the major source of energy for embryos during incubation. Digestion of these lipids is catalyzed by lipases secreted from the internal surface of the yolk sac of the embryo (Romanoff, 1969) and it appears that pancreatic and biliary secretions are not required for yolk utilization. Lipase activity increases with incubation time in turkey eggs (Escribano *et al.*, 1988). Total lipase activity/yolk membrane increases from 31 $\mu\text{eq}/\text{min}$ at day 9 of incubation to a maximum of 214 $\mu\text{eq}/\text{min}$ at day 21. This is probably due to the switch from albumen to yolk as the major energy source. The role of the liver and pancreas in digestion only becomes important after the bird starts to eat (Romanoff, 1969).

2.4.1.2 Fat digestion after hatching

Fat digestion improves with age after hatching with the effect most pronounced for animal fats containing saturated fatty acids (Salmon, 1977; Polin and Hussein, 1982; Sell *et al.*, 1986). In poults, utilization of tallow between 2 and 8 weeks of age increased from 57 to 74% (Sell *et al.*, 1986). This improvement was mainly related to increasing digestibility of saturated fatty acids. The improvement in fat utilization is also reflected by the metabolizable energy of feed containing tallow which increased from 28.2 MJ/kg at 2 weeks to 38.2 MJ/kg at 8 weeks of age for poults. The digestibility of mutton tallow by chicks was lower at early ages while the utilization of the more unsaturated linseed oil was not (Duckworth *et al.*, 1950). Intestinal absorption of 20% beef tallow was studied in egg-type chicks during the period of 2 to 7 and 8 to 15 days of age (Carew *et al.*, 1964). They showed fat absorption increased with age from 40 to 79%.

2.4.1.2.1 Effect of rate of feed passage

It has been speculated that feed transit time in the digestive tract may affect fat digestion in young birds (Polin and Hussein, 1982). The lower fat digestion seen in young birds was thought to be related to a rapid transit time and that as transit time decreased with age, fat digestibility increased (Golian and Polin, 1984). However, research on the effects of age on feed transit time are

contradictory which reduces the credibility of this explanation (Noy and Sklan, 1995). In addition, longer intestinal transit times have been associated with microbial proliferation in the small intestine which leads to bile salt deconjugation and lower fat absorption (Bali *et al.*, 1988; Salih *et al.*, 1991). In conclusion, feed transit time is unlikely to be the major or only factor affecting fat digestion in the young bird.

2.4.1.2.2 Bile salts status

Bile salt status in birds changes with age. The improved utilization of fats in older birds may be related to an enhanced synthesis rate of bile salts and/or a more efficient enterohepatic circulation of bile salts (Smallwood *et al.*, 1970, 1972; Jackson *et al.*, 1971). Better fat utilization of fats in older birds may also be related to the balance between bile salt concentration in the gut and pancreatic secretions. Compared to new-born mammals (Sewell *et al.* 1982), a relatively high concentration of bile salts in 2-day old chickens in jejunal contents (13.9 vs 2 mM/l) was shown by Green and Kellogg (1987). It decreased at day 9 by 8.2 mM/l and again increased to 13.7 and 20.5 mM/l by day 16 and 23 respectively. It is also suggested that young chicks have a low replacement rate for bile salt lost by excretion compared to older birds (Serafin and Nesheim, 1970; Green and Kellogg, 1987). Since high concentrations of bile salts interfere with lipase action during fat digestion process, and if this effect coupled with lower lipase secretion at this

time, it is possible that lower fat utilization in younger birds may relate to the bile salt concentrations during early ages.

2.4.1.2.3 Digestive enzymes status

Numerous reports have shown that the concentration of digestive enzymes in the small intestine of poultry change with age (Krogdahl and Sell, 1989; Brannon, 1990). Duodenal activity of trypsin, amylase and lipase in young chicks increased by 100, 50, and 20 fold respectively between 4 and 21 days of age (Noy and Sklan, 1995). Lipase activity of the pancreas and intestinal contents of turkeys also increases with age (Krogdahl and Sell, 1984, 1989). Lipase activity was relatively low in newly hatched birds, increased slowly to about 6 weeks of age and then increased rapidly to 8 weeks of age. Similarly, Escribano *et al.* (1988) reported that the total lipase activity in the pancreas of young turkeys averaged 400 $\mu\text{eq}/\text{min}$ at day 1 and increased exponentially to 8400 $\mu\text{eq}/\text{min}$ by day 16 after hatching

Development of intestinal lipase activity seems to be dependent on dietary fat level. Low activities were observed when low fat diets were fed throughout the study (Krogdahl and Sell, 1989). With high fat diets, a lag period of about 3 weeks was followed by a 5 fold increase in lipase activity (Fig. 2.1). It is

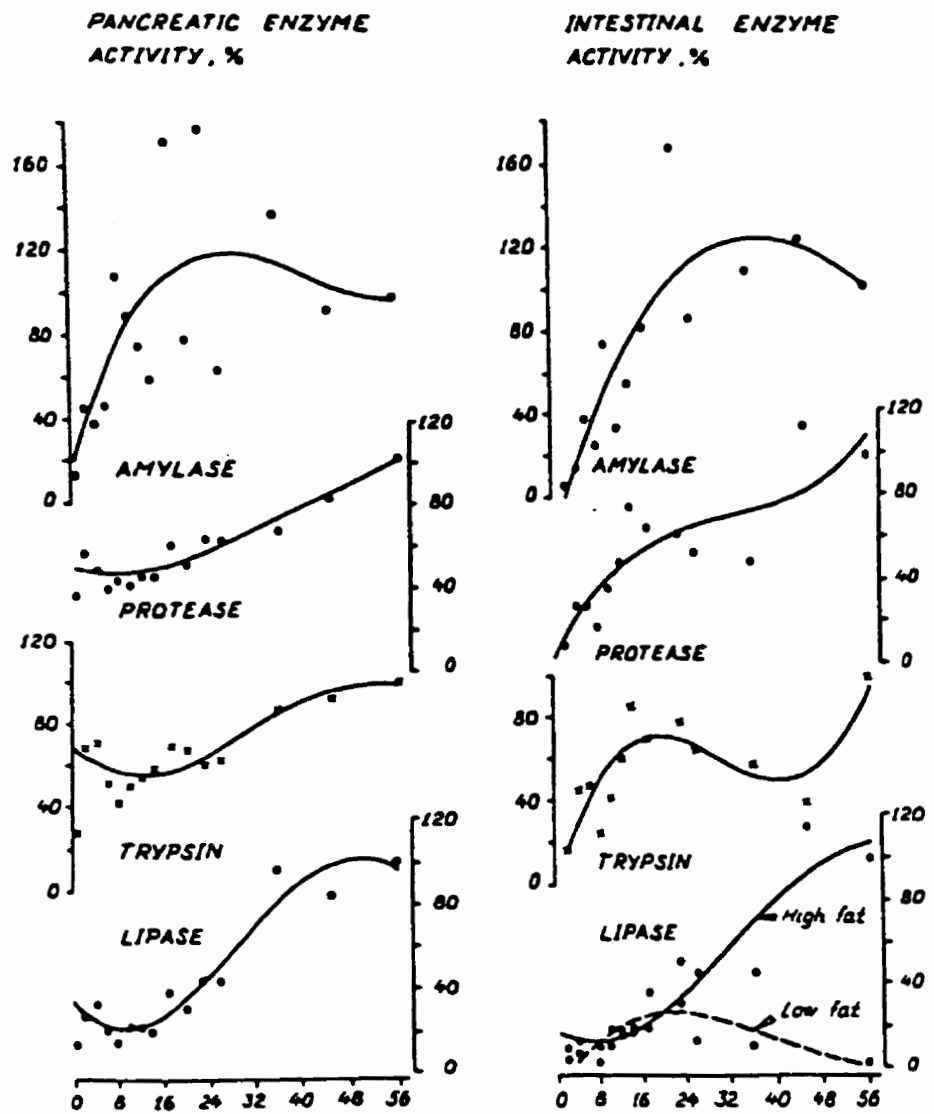


Figure 2.1. Development of digestive enzymes in pancreatic tissue and intestinal contents of poult with age (From Krogdahl and Sell, 1989).

suggested that lipase insufficiency may be a limiting factor for lipid digestion in young chicks and poults (Sell *et al.*, 1986).

2.5 Dietary Supplements

It is obvious that digestion and absorption of fat is complex but bile salt and lipase insufficiency have received the most research attention in relationship to steatorrhoea in young birds. This research has focused on long chain, saturated fatty acids because their utilization is most affected by age.

2.5.1 Dietary bile salt supplements

The possibility of improving fat utilization by using dietary bile salts has been studied extensively. Apparent fat absorption from 1 to 2 weeks of age increased from 47 to 69% when chicks were fed 0.5% ox bile in a diet containing 20% tallow (Fedde *et al.*, 1960). Addition of 0.25% chenodeoxycholic acid to a diet containing 8.3% tallow caused a larger increase in fat absorption in 4 to 7 day-old chicks (39.6 vs 51.2%) compared to control diet (Gomez and Polin, 1976). Addition of 0.25% cholic acid, the most prevalent bile acid found in the chicks (Webling and Holdsworth, 1965), caused minor improvement in fat absorption (39.6 vs 46.9). Conjugated taurodeoxycholate at the 0.25% level at this age had no effect of fat absorption but higher levels (0.5%) increased fat absorption (39.6 vs 48.4%). Absorption of fats in broiler chicks, 14 to 19 days of age, fed a diet

containing 8% tallow improved from 68.2% to 74.6% with the addition of dietary cholic acid. Cholic acid improved the metabolizable energy (ME) of tallow in young chicks but not in laying hens (Gomez and Polin, 1976). They stated that older chickens have no problem with digestion and absorption of these lipids with absorption rates of 85 to 89%.

The effect of Na-taurocholate supplementation in tallow-based diets in three different breeds of chickens was studied by Katongle and March (1980). They found fat absorption was significantly increased in broilers and White Leghorn chicks supplemented with 0.05% Na-taurocholate, but not for New Hampshire chicks. Eight-week-old chickens retained 43-63% of the tallow consumed when their bile ducts were cannulated compared to 90-92% for sham operated birds (Pullen and Polin, 1984). Garret and Young (1964) cannulated bile ducts of 8 to 10 week-old chicks and observed that in the absence of bile, 36 to 50% of fatty acids were absorbed indicating that the lipid-bile salt micelle is not the only prerequisite in the process of fatty acid digestion and absorption.

Bile salts have the potential to improve the process of fat digestion and absorption as seen in different studies in young birds. However, the increase in the utilization of the tallow does not fulfil the potential which is present in the older birds (85-89%). The small improvement (5-20%) in the absorption of tallow as a result of the addition of bile salts to the diet demonstrates that they do not rectify the poor fat utilization in young birds.

2.5.2 Lipase supplements

Dietary lipase supplements have been used extensively in humans to prevent steatorrhoea in cases of pancreatic insufficiency (DiMagno *et al.*, 1973, 1975; Graham, 1977; Regan *et al.*, 1977). However, research on the use of dietary lipase for poultry species is restricted to a study by Polin *et al.* (1980). They examined the effect of crude porcine pancreatic lipase (0, 0.01 or 0.1%) in combination with bile salts (cholic acid, chenodeoxycholic acid, dehydrocholic acid, deoxycholic acid or Na-taurocholate) on fat digestibility of a corn-based diet containing 4% tallow by White Leghorn males. Lipase supplemented at 0.1% from 2 to 9 days after hatching numerically improved fat absorption either alone or in combination with 0.4% cholic acid but the effect was not significant. All other treatments failed to improve fat absorption.

The failure of the pancreatic lipase to improve the fat digestibility of chicken diets should not be unexpected. *In vitro* and *in vivo* (human) studies have shown that porcine lipase denatures in acidic pH and therefore the desirable activity does not reach the site of digestion in the duodenum (Go *et al.*, 1970; DiMagno *et al.*, 1977). In addition, porcine lipase requires colipase for maximal activity in the presence of bile salts and colipase is also denatured by acidic pH (DiMagno *et al.*, 1977). It can be speculated that lipase sources with better stability in low pH and less dependency on colipase and bile salts would be more likely to improve fat digestibility when added as diet supplements.

2.6 Bile Salt-Lipase-Colipase Interactions

Hydrolysis of triglycerides by pancreatic lipase is strongly inhibited by bile salts present in the duodenum and colipase is the only agent known to counteract this inhibition. Lipase, by itself, has affinity only towards hydrophobic phases and can not act on hydrophilic substrates (Sternby and Borgstrom, 1979). In the presence of bile salts, binding to the substrate interface is pH dependent. Bile salts present at a level equal to or greater than the critical micellar concentration (CMC) in a media of greater than pH 6.5 displace lipase from its binding site resulting in a reversible inactivation (Bosc-Bierne *et al.*, 1984). Ouagued *et al.* (1980) stated that the presence of excess colipase is necessary for measuring lipase activity. Addition of increasing amounts of colipase progressively increased lipase activity in rat. There was a good linear relationship between colipase content of the sample and lipolytic activity. From extensive studies of pancreatic enzymes, it is known that optimum activity and assay linearity are obtained only if both bile salts and colipase are included in the assay (Borgstrom and Erlanson-Albertson, 1973; Mourot and Corring, 1979; Brockman, 1981). Addition of colipase to the bile salt-lipase-substrate system, has a dramatic effect on the binding of lipase to the substrate interface and therefore lipase activity. Some detergents such as dodecylsulphate and sulphonate don't act like bile salts and addition of colipase does not reverse the inactivation of lipase. This may be due to enzyme denaturation by these detergents (Brockerhoff, 1971).

2.7 Fat Utilization and Trypsin

Green and Nasset (1980) stated that bile salts stabilize intestinal enzymes. Diversion of bile from the intestine of rats for one day decreased intestinal trypsin and chymotrypsin activity, but increased enzyme secretion. They showed that bile salts in the small intestine control the rate of disappearance of intraluminal trypsin and chymotrypsin by inhibiting their auto digestion. Rinderknecht (1986) in an *in vitro* study showed that the activity of trypsin was increased by low levels of bile salts. Trypsin is a key enzyme for activation of pro-colipase to colipase (Colomb *et al.*, 1979; Borgstrom *et al.*, 1979a, b; Bosc-Bierne *et al.*, 1984; Rinderknecht, 1986). Addition of trypsin into an assay system shortens the time of fat hydrolysis. In a kinetic study, Bosc-Bierne *et al.* (1981) found lag times of about 50 min in the presence of pro-colipase. The lag time represents the time needed by the enzyme to bind onto fat droplets and to reach the maximal rate of triglyceride hydrolysis (Borel *et al.*, 1994). Lag time was shortened to 5 min after the cofactor was treated with 1% trypsin at pH 8 prior to addition to the assay system. Similar results were obtained by Borgstrom *et al.* (1979b) when they dissolved lyophilized chicken pancreatic juice in distilled water and measured lipase activity. When the pancreatic juice was treated with trypsin, the lag time of lipid hydrolysis was decreased from 50 to 4 minutes indicating that trypsin had changed pro-colipase to colipase.

2.8 Lipid-Bile Salt-Cholecystokinin Interactions

Studies strongly support the concept that bile acids decrease cholecystokinin (CCK) release and thereby affect other aspects of fat digestion (Bullock *et al.*, 1991; Green, 1994). Thomas and Crider (1943) demonstrated that bile in the intestine had an inhibitory effect on nutrient stimulated pancreatic secretions in the dog. Malagelada and co-workers (1973, 1976) demonstrated in healthy human subjects that bile acids at physiological concentrations (critical micellar concentration, CMC) could inhibit or abolish gall bladder contraction or pancreatic enzyme secretion stimulated by luminal fatty acids or amino acids. Intraduodenal infusion of sodium taurocholate decreased pancreatic secretions (Fig. 2.2, indicated by trypsin output) and gallbladder contraction (Fig 2.3, indicated by bilirubin output). Green (1994) showed that this inhibitory effect was due to a decrease in CCK release. The important functions of CCK are stimulation of pancreatic enzyme secretion, pancreatic growth, pancreatic HCO_3^- secretion, gallbladder contraction and inhibition of gastric emptying (Hunt and Groff, 1990; Bullock *et al.*, 1991). Cholecystokinin inhibits gastric emptying probably by initiating the vagovagal reflex which results in inhibition of both the proximal and distal stomach in rats and dogs (Yamagishi and Debas, 1978) causing relaxation of the body of the stomach (Raybould and Tache, 1988). Cholecystokinin also plays an important physiological role in the regulation of acid secretion in humans, rats and dogs (Moran and McHugh, 1982; Jin *et al.*, 1994). Cholecystokinin inhibits

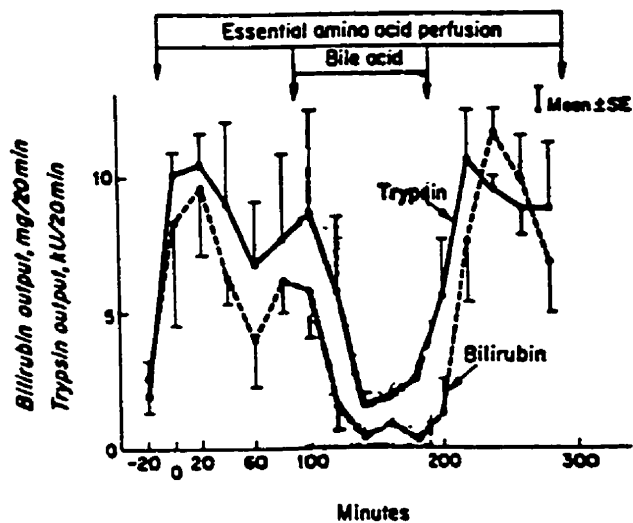


Figure 2.2. Trypsin and bilirubin outputs during continuous intraduodenal perfusion of EAAs before, during and after addition of sodium taurocholate in human subjects (From Malagelada *et al.*, 1973)

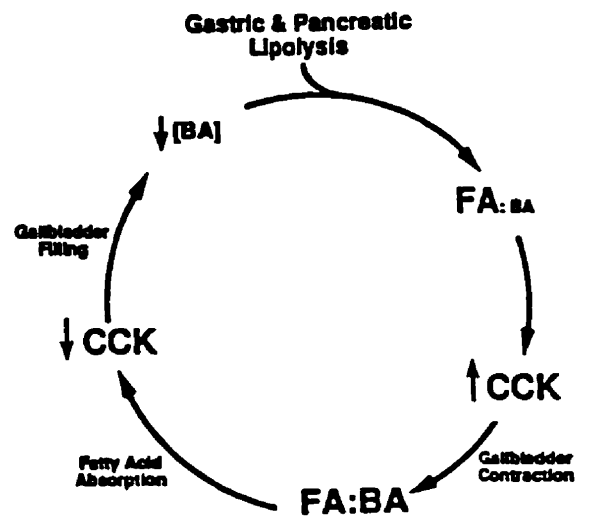


Figure 2.3. Schematic diagram illustrated the relation between the ratio of fatty acids to bile acids (FA:BA) in the intestine on CCK release, FA absorption and gallbladder contraction. (From Green, 1994)

acid output in the dog when administrated intravenously in a dose of 56 pmol/kg/hr (Jin *et al.*, 1994). The inhibitory effect of bile salts on pancreatic secretions and the schematic diagram of this effect on CCK release are presented in Figures 2.2 and 2.3.

2.9 Type of Feed and Bile Excretion

Fat type affects bile excretion with the effect being fatty acid dependent. Unsaturated fatty acids increase while saturated fatty acids decrease excretion of bile salts in adult Single Comb White Leghorn cockerels (Lindsay *et al.*, 1969). Unsaturated fatty acids also increase excretion of bile acids in humans (Haust and Beveridge, 1958; Goldsmith *et al.*, 1960; Moore *et al.*, 1962). Ingestion of diets high in unsaturated fatty acids usually cause a decrease in the levels of cholesterol and other lipids (LDL, HDL, VLDL) in serum (Kinsell *et al.*, 1954). One mechanism which could decrease serum-cholesterol concentration would be an increase in fecal excretion of sterols and bile acids (Goldsmith *et al.*, 1960). This may also be related to an acceleration of the conversion of cholesterol to cholic acid.

2.10 Use of Enzymes in Pancreatic Insufficiency

The use of orally supplemented pancreatic enzymes, especially lipase, has with the exception of one report (Polin *et al.*, 1980) not been reported in poultry

studies. Therefore, the following results are mostly based on studies with human subjects.

A reduction in enzyme output from the pancreas results in progressively more severe malabsorption and causes steatorrhoea. Although it would appear that oral administration of supplemental pancreatic enzymes should correct the enzymatic deficiency, it has often not been as effective as expected (DiMagno *et al.*, 1973). Among the factors which affect the efficacy of pancreatic enzyme replacement therapy, the following factors are most important: 1. Destruction of pancreatic enzymes in the acid environment of stomach; 2. the variability in the potency of commercial pancreatic enzyme supplements; 3. loss of pancreatic enzymes during aboral small intestinal transit; 4. the possible loss of enzyme activities during freezing; 5. the possible loss of enzyme activities prior to consumption.

2.10.1 Denaturation of pancreatic enzymes by acid

Oral pancreatic enzyme replacement therapy is routinely used for malabsorption in pancreatic insufficiency (Warren *et al.*, 1967; Saunders and Wormsley, 1975). Due to inactivation of lipase and trypsin by gastric acid and pepsin, treatment with enzymes usually, has not been successful (Go *et al.*, 1970; DiMagno *et al.*, 1977). Inactivation of the enzymes before they reach the small intestine is an important aspect of oral enzyme replacement therapy for treatment

of malabsorption (DiMagno *et al.*, 1977). The role of pepsin in inactivation of enzymes has been demonstrated by Heizer *et al.* (1965). They showed that trypsin was inactivated by pepsin and acid while lipase was inactivated by a pH of less than 4 in human subjects. DiMagno *et al.* (1977) also showed that trypsin and lipase can be inactivated by pepsin and acid in the stomach.

2.10.1.1 How to protect enzymes from acidity

2.10.1.1.1 Use of antacids or inhibition of gastric acid secretion

Either neutralization of gastric acidity or inhibition of gastric acid secretion should improve the efficacy of pancreatic enzyme therapy (Graham, 1977). Graham (1982) investigated the effect of the addition of sodium bicarbonate, aluminium hydroxide, magnesium hydroxide and calcium carbonate as antacids, or cimetidine as an inhibitor of gastric acid secretion to supplemental pancreatic enzymatic therapy in the human patients with severe exocrine pancreatic insufficiency. He found that gastric acidity is the major factor preventing enzymatic activity in the duodenum. Pancreatic enzymes, in particular lipase, are rapidly and irreversibly inactivated by acid conditions. The co-ordination of enzyme tablets with either sodium bicarbonate or aluminium hydroxide caused a greater reduction in steatorrhoea than enzymes alone. The other antacids were not effective. Regan *et al.* (1978) suggested that antacids are not effective because

they can not provide sufficient protection against the hostile environment of the stomach and they stimulate the stomach to secrete gastric acid.

Although Graham (1982) found cimetidine was not effective for improving activity of lipase taken orally, previous work demonstrated a beneficial effect (Regan *et al.*, 1977, 1978). The latter reports associated the usefulness of cimetidine to its potent anti-secretory effects which caused increased gastric and duodenal pH, decreased gastric and duodenal volumes and increased delivery of lipase in concentrations of greater than 5% of normal into the duodenum. A pancreatic enzyme output of less than 10% of normal is associated with steatorrhoea (DiMagno *et al.*, 1975). Combined oral administration of cimetidine and pancreatin reduced steatorrhoea (Regan *et al.*, 1977). They concluded that cimetidine protects orally ingested trypsin and lipase from acid-peptic inactivation and thereby increases enzyme concentrations within the duodenal lumen. They emphasised that steatorrhoea is inversely related to duodenal lipase recovery.

Although the use of cimetidine or some of the other mentioned compounds may not be practical in the poultry industry, understanding their mode of action may help to increase the potential for dietary enzyme supplements.

2.10.1.1.2 Enteric-coated method

Older studies with enteric-coated pancreatic enzyme preparations were ineffective in overcoming pancreatic insufficiency (Goodchild *et al.*, 1974;

Graham, 1977). It is likely that these capsules released the enzyme contents in the stomach, where they were inactivated by acid and pepsin, or the capsule did not or was slow to dissolve in the small intestine (DiMagno *et al.*, 1973, 1975). However, more recent work has shown that an enteric-coated microsphere formulation of pancreatin greatly improved the efficacy of pancreatic enzyme replacement in the treatment of pancreatic steatorrhoea (Roberts, 1989). This coating protected the acid sensitive enzyme from degradation of acidic conditions of the stomach. The small size of the microspheres compared with the older enteric-coated tablets allowed for their rapid passage through the pylorus together with the meal. The enteric coating was designed to dissolve only in the more neutral duodenal contents, thus releasing the enzymes into an environment where they can act. Although coating pancreatin protects it from degradation in the stomach, the coating needs about 30 minutes time to dissolve at pH 6 (Littlewood *et al.*, 1988) and so still causes a delay in enzyme release in the duodenum.

2.10.1.1.3 Use of acid-stable lipases

In pancreatic lipase insufficiency, enzyme replacement therapy with acid-stable lipases has been suggested as an alternative method for steatorrhoea treatment (Abrams *et al.*, 1984). Microbial lipases have received attention in recent years because they show a wide range of characteristics including more

stability in acidic conditions. In the following sections, some of their properties are discussed

2.10.2 Variability in pancreatic enzyme supplements

In addition to porcine lipase, some microbial enzymes, in particular *Aspergillus*, *Rhizopus* (fungal lipases) and *pseudomonas* lipase (bacterial lipase), have been used for treatment of steatorrhoea in humans suffering from pancreatic insufficiency (Canioni *et al.*, 1977; Schneider *et al.*, 1985; Moreau *et al.*, 1988; Raimondo and DiMagno, 1994). Research work with lipases has markedly increased in the last decade due to their potential use in the food, detergent and medical industries. Isolation and purification of lipases from different sources such as animals, plants and micro-organisms have been extensively reported (Taipa *et al.*, 1994). Microbial lipases have a wide range of enzymatic properties and substrate specificities. They have been attractive because they are easily produced in large amounts for industrial applications (Pokorny *et al.*, 1994).

2.10.2.1 Selectivity of lipases

Selectivity is one of the characteristics of lipases that controls the digestion of triglycerides and the absorption of the resulting fatty acids and monoglycerides. This property in some sources of lipase is shown in Table 2.2. Mammalian lipases are specialized to hydrolyze 1 and 3 position of triglycerides. Fungal lipases have

Table 2.2. Substrate specificity of lipases sources

Lipase source	Substrate specificity	Reference
Mammalian lipase		
Pancreatic	1 and 3 position of triglycerides	Rinderknecht, 1986
Gastric	1 and 3, shorter chain (C8:0-C12:0)	Jensen <i>et al.</i> , 1994
Fungal lipase		
<i>Aspergillus niger</i>	Diglyceride	Zentler-Munro <i>et al.</i> , 1992
<i>Candida cylindracea</i>	Broad (1,2 and 3)	Benzonana and Esposito, 1971
<i>Geotricum candidum</i>	Cis-9 unsaturated fatty acids	Sonnet <i>et al.</i> , 1993
<i>Penicillium cyclopium</i>	Broad	Okumura <i>et al.</i> , 1976
<i>Penicillium camembertii</i>	Only mono and diglycerides	Yamaguchi and Mase, 1991
Bacterial lipase		
<i>Chromobacterium viscosum</i>	Broad	Yamaguchi <i>et al.</i> , 1973; Taipa <i>et al.</i> , 1994
<i>Pseudomonas sp.</i>	Broad	Nishio <i>et al.</i> , 1987; Aoyama <i>et al.</i> , 1988
<i>Pseudomonas aeruginosa</i>	Broad	Stuer <i>et al.</i> , 1986
<i>Pseudomonas capacia</i>	Broad	Jorgensen <i>et al.</i> , 1991
<i>Pseudomonas fragi</i>	1 and 3 preferably long chain fatty acids	Lawrence <i>et al.</i> , 1967
<i>Pseudomonas fluorescense</i>	Broad	Sugiura <i>et al.</i> , 1977
<i>Pseudomonas glume</i>	Broad	Frenken <i>et al.</i> , 1992

a broad range of selectivity including specificity for positions 1 and 3. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triglyceride (Jaerger *et al.*, 1994). In principal, microbial lipases with specificity for fatty acids in the 1, 3 positions of triglycerides could be used to produce 2 monoglycerides (Stead, 1986).

2.10.2.2 Fungal lipases

Several fungi produce an acid-resistant lipase. The clinical efficacy of a fungal lipase depends at least to some degree on its characteristic for survival and activity in the stomach (Zentler-Munro *et al.*, 1992). Enzymes produced by *Rhizopus arrhizus* and *Aspergillus oryza* have shown promising efficacy when compared with a conventional pancreatin formulation (Schneider *et al.*, 1985). Zentler-Munro *et al.*, (1992) in an *in vitro* study showed that *Aspergillus niger* lipase has a wide pH range of 2.5 to 5.5. The latter authors showed that the pH optimum of *Aspergillus niger* lipase is approximately 4.5 and inhibition below this pH is completely reversible. *In vitro* study showed that *Aspergillus niger* lipase generates mainly diglyceride rather than monoglyceride (Zentler-Munro *et al.*, 1992). Although *Aspergillus niger* was shown to be pepsin and trypsin resistant, no beneficial effect was found by using this enzyme in human subjects suffering from steatorrhoea (Zentler-Munro *et al.*, 1992). This may be because *Aspergillus niger* fungal lipase is inhibited by bile acids at the concentration found in the

duodenum. *In vitro* study showed that *Rhizopus* lipase does not need colipase (Canioni *et al.*, 1977). Moreau *et al.* (1988) found that *Rhizopus* fungal lipase is not resistant to pepsin. More research is required to understand the sensitivity of microbial lipases to trypsin and chymotrypsin.

2.10.2.3 Bacterial lipases

Pseudomonas lipase has potential industrial application because it is stable at high temperatures (Iizumi *et al.*, 1990) and in the presence of organic solvents. Frenken *et al.* (1992) indicated that the lipases of *Pseudomonas* have significant overall sequence similarity. *Pseudomonas* lipases with the exception of *Pseudomonas fluorescence* also have similar tertiary structure (Duong *et al.*, 1994). The pH and temperature optimum of many strains of *Pseudomonas* are in the range of 7-9 and 30-50 °C respectively (Stead, 1986). Some of the *Pseudomonas* lipases from *P. fluorescence*, *P. putida*, *P. fragi* and *P. aeruginosa* have a detrimental effect on stored food products because they are active psychrotrophic bacteria that are able to grow and hydrolyze triglycerides at 7 °C or below (Stead, 1986). Raimondo and DiMagno (1994) showed that bacterial *Pseudomonas glumae* lipase, in contrast to porcine lipase, does not need colipase for activity and is not inhibited by bile acids. They concluded that bacterial lipase lipolytic activity is much more resistant to inactivation than the lipolytic activity of porcine lipase. Bacterial lipolytic activity should survive much better within the

gastrointestinal lumen than the lipolytic activity of either human or fungal lipase. Human lipase loses 80% of its lipolytic activity during small intestinal transit (Layer *et al.*, 1986). Fungal lipases are inactivated in the presence of bile acids (Schneider *et al.*, 1985; Zentler-Munro *et al.*, 1992). Use of bacterial lipase greatly simplifies the treatment of pancreatic steatorrhoea by eliminating the need for enteric-coating or the ingestion of acid-neutralizing or anti secretory drugs (Raimondo and DiMagno, 1994).

2.10.3 Pancreatic enzyme status during intestinal transit

The activity of pancreatic enzymes in chyme decreases during transit from the proximal to the distal intestine. Low (1982) showed that pancreatic proteases and lipase in growing pigs were rapidly inactivated intraluminally. Similar results were found in rats (Pelot and Grossman, 1962). Activities of all pancreatic enzymes decrease progressively and at similar rates as they move from the proximal to the distal small intestine (Borgstrom *et al.*, 1957). In contrast Layer *et al.* (1986) showed that activities of lipase, trypsin and amylase in healthy humans decreased at very different rates as chyme travelled from the duodenum to the ileum. Of these enzymes, lipase activity decreased (95%) most rapidly during small intestinal transit. Trypsin activity survived significantly better than lipase. In contrast to lipase and trypsin, most amylase activity remained. The greater survival of amylase in the human small intestine is probably because of its

resistance to enzymatic proteolysis (Granger *et al.*, 1975). Inactivation of ingested lipase can occur in the stomach due to an acid dependent degradation process, or continue through the small intestinal transit as an acid independent degradation process (Layer *et al.*, 1986). Chymotrypsin, trypsin and lipase in human duodenal contents lose their activities when incubated at 37 C (Thiruvengadam and DiMagno, 1988). The loss of enzyme activity was greatest for lipase and after 2 hours only 24% of initial lipase activity remained. The loss of lipase activity was affected more by chymotrypsin than by trypsin. Addition and inhibition of chymotrypsin rapidly accelerated and abolished the loss of activity, respectively. They concluded that chymotrypsin is a major factor causing loss of lipase activity. Chymotrypsin alone was sufficient to destroy lipase activity, whereas trypsin in the absence of chymotrypsin does not affect lipase activity. Thus lipase is relatively more resistant to trypsin digestion and trypsin digestion of lipase requires the presence of chymotrypsin. Similarly, Bousset-Risso *et al.* (1985) found that chymotrypsin inhibits porcine lipase more than trypsin. They stated that chymotrypsin splits porcine lipase at the Phe. 335-Ala. 336 bond. De Caro *et al.* (1986) suggested that the 336-449 fragment is part of the active site of lipase and proteolysis by chymotrypsin at or near the active site of lipase can inactivate lipase. Proteolytic digestion and inactivation of lipase activity during small intestinal transit has important physiological and pathophysiological consequences (Thiruvengadam and DiMagno, 1988).

An *in vitro* study by Kelly *et al.* (1988) showed that triolein and casein significantly increased the survival of lipase activity. The effect of nutrients on the survival of lipase is dependent on the initial concentration of lipase. Casein and rice starch significantly affected the survival of trypsin and chymotrypsin (Kelly and DiMagno, 1989). They showed that casein maintained lipolytic activity even though survival of chymotrypsin activity was also enhanced. It is suggested that nutrients provide an alternate substrate for chymotrypsin, replacing lipase in that role (Muller and Ghale, 1982; Thiruvengadam and DiMagno, 1988).

2.10.4 Loss of enzyme activities during freezing

The activity of pancreatic enzymes in duodenal juice is usually used to estimate the status of pancreatic enzyme secretion. Since it is common practice to freeze samples before assay, loss of enzyme activity during freezer storage may have an unrecognized impact on these studies (Kelly *et al.*, 1991). Proteolytic activity, trypsin and chymotrypsin, is more stable than lipolytic activity in duodenal juice during *in vitro* incubation (Thiruvengadam and DiMagno, 1988). Kelly *et al.* (1991) postulated that casein protected lipase during freezing. They concluded that either lipolytic activity in duodenal juice should be assayed immediately, or if juice is frozen, casein (33.5 mg/ml as a final concentration) or turkey egg white (5 mg/ml as a final concentration) should be added to protect lipolytic lipase activity from chymotrypsin digestion. Turkey egg white (TEW)

selectively inhibits chymotrypsin (Hotz *et al.*, 1983). After storage of duodenal contents for a period of 56 days, 80% of chymotrypsin activity was inhibited by 5 mg/ml turkey egg white (Kelly *et al.*, 1991). Time of storage had no effect on chymotrypsin inhibition. Thiruvengadam and DiMagno (1988) showed that TEW decreased chymotrypsin activity significantly during a 120 min test incubation at 37 C from 11318 to 1954 unit/min/ml. It is probable that casein or turkey egg white provide an alternate substrate for chymotrypsin, replacing lipase in that role. Corn oil added to duodenal juice also protected lipase during freezing (Muller and Ghale, 1982).

2.10.5 Enzyme status prior to consumption

Enzymes that are to be used in animal feeding must survive storage and the feed manufacturing process (Classen and Bedford, 1991; Campbell and Bedford, 1992). Most enzymes have been shown to be relatively stable to storage in the dry form so long as high temperatures and humidities are avoided (Campbell and Bedford, 1992). The exposure to moisture and temperature during feed processing (eg. pelleting) has the potential to damage enzymes with the degree of effect dependent on enzyme type and source. Selection of stable enzymes and precise control of manufacturing conditions are required to prevent denaturation of enzymes prior to consumption by animals.

2.11 Concluding Remarks

Fat utilization, in particular saturated fatty acids, is not developed well in very young chicks and it seems that the major factor involved in this problem is the lipase deficiency. The virtual lack of poultry research in this area means that considerable preliminary research must be completed before successful industrial application. Preliminary *in vitro* studies reported here were based on selecting the best lipase from selected lipases in different environmental conditions associated with the proventriculus and small intestine of young birds. Seven lipase sources, *Rhizopus arrhizus*, and *Aspergillus niger* (fungal), two *Pseudomonas sp.* and *Chromobacterium viscosum* (bacterial) and crude and lyophilized porcine lipase (mammalian) were evaluated. The lipase that showed the best response to different conditions in *in vitro* studies was chosen for *in vivo* experiments in broilers and turkey poults

3.0 STABILITY OF PORCINE AND MICROBIAL LIPASES TO CONDITIONS WHICH APPROXIMATE THE PROVENTRICULUS OF YOUNG BIRDS

3.1 Abstract

In vitro experiments were conducted to characterize the activity and the stability of lipase from animal (crude porcine, CPL; lyophilized porcine, LPL), fungal (*Rhizopus arrhizus*, RAL; *Aspergillus niger*, ANL), and bacterial (two *Pseudomonas sp.*, PL1, PL2; and *Chromobacterium viscosum*, CVL) sources when exposed to conditions associated with the glandular stomach. Lipase activity was measured at pH ranges of 3 to 8, 40 C and then monitored in response to time of exposure (0 and 30 minutes), pH (3 and 7) and pepsin level (5, 50 and 500 U/ml). All lipases except ANL and CVL had maximum activity at pH 7-8. The optimal pHs for ANL and CVL were 5 and 6-8, respectively. Exposure of lipases to 40 C and pH 7 for 30 minutes reduced the activity of all lipases except ANL. In contrast, 40 C increased ANL activity 2.5 fold. Although activity of all lipases was reduced by exposure to pH 3, it was nearly eliminated for CPL and LPL. Pepsin concentration had only minor effects on lipase activity and then only at high concentration. The results demonstrate that bacterial lipases (PL1, PL2 and CVL) and ANL are more stable under conditions that approximate the glandular

stomach and may explain why dietary porcine lipase has been ineffective in preventing fat malabsorption in previous *in vivo* studies.

3.2 Introduction

Studies on the digestibility of fats by chickens and turkeys have shown that the utilization of saturated fats is lower in young poults (Sell *et al.*, 1986) and poults (Leeson and Atteh, 1995) than in the mature birds. The reasons for poor fat digestibility are not resolved but may reflect the underdeveloped state of gastrointestinal functions at the time of hatch. Potential reasons are a rapid feed transit time (Vergara *et al.*, 1989), and low levels of bile salt (Krogdahl and Sell, 1984) and pancreatic secretions (Noy and Sklan, 1995).

Relative to the adult, a lower digestibility of a given nutrient will occur in the young bird if the process of digestion and absorption is not complete during the shortened period of gastrointestinal passage. Additives that improve the efficiency of triglyceride digestion and absorption have the potential to increase fat digestibility in the young bird. In the young bird, bile salt secretion is lower and enterohepatic circulation of bile salts is higher than occurs in the adult (Jackson *et al.*, 1971; Green and Kellogg, 1987). However dietary supplementation with lower amount of bile salts has resulted in only a small change in fat utilization (Gomez and Polin, 1976). In comparison with the digesta of humans and rats (Watkins, 1975), relatively high concentrations of bile salts (2 vs 14 mM/L) have

been found in the 2-day old chick (Green and Kellogg, 1987). These observations suggest that bile salt insufficiency may not be the primary cause of poor fat utilization in the young birds (Gomez and Polin, 1974, 1976).

Numerous reports indicate that the concentrations of digestive enzymes in poultry increase with age (Krogdahl and Sell, 1989; Pubols, 1991; Noy and Sklan, 1995). Duodenal activity of lipase in young chicks increases 20 times between 4 and 21 days of age (Noy and Sklan, 1995). In turkeys, the lipase activities of the pancreas and intestinal contents were relatively low in the newly hatched bird, increased slowly to about 6 weeks of age, and then increased rapidly by 8 weeks of age (Krogdahl and Sell, 1984, 1989). The relatively low rate of pancreatic lipase secretion in young birds may be insufficient for substantial triglyceride hydrolysis and therefore could contribute to poor fat utilization.

Dietary supplementation with lipase has the potential to improve fat utilization in situations of pancreatic lipase insufficiency. In humans pancreatic enzyme replacement therapy is used routinely to treat malabsorption (Saunders and Wormsley, 1975). However dietary supplementation with pancreatic lipase results in only a partial improvement in fat digestibility (DiMagno *et al.*, 1973).

The use of dietary supplemented pancreatic lipase, has with the exception of one paper (Polin *et al.*, 1980) not been reported in poultry studies and most results are based on pancreatic insufficiency disease in humans. Supplementation of crude porcine lipase (steapsin) in a corn-soy based diet containing 4% tallow

was tested in young chicks but did not produce beneficial effects on fat utilization or bird performance.

Destruction of pancreatic enzymes in the acid environment of the stomach (DiMagno *et al.*, 1977), the variability in the potency of commercial pancreatic lipase supplements (Raimondo and DiMagno, 1994), and loss of pancreatic enzymes during aboral small intestinal passage are possible reasons as to why supplemental pancreatic lipase is ineffective or less effective than anticipated.

Dietary lipase can be protected from the adverse conditions of the gastric stomach by a number of methods. Gastric pH can be increased by the use of antacids or cimetidine which inhibits gastric acid secretion (Graham, 1977). Lipase can be protected by the use of enteric coating procedures (Roberts, 1989). Another possibility is to use enzyme sources which are stable under conditions of the stomach.

No information is available as to the relative stability of various lipase sources under condition of the passage through the proventriculus in young birds. The objective of this study was to evaluate the activity and stability of lipase sources after exposure to conditions that approximate passage through the proventriculus.

3.3 Materials and Methods

A total of seven sources of lipase was used in this study. Crude porcine lipase (CPL), lyophilized porcine lipase (LPL, 50 KDa), and *Rhizopus arrhizus* lipase (RAL, 40 KDa), were obtained from Sigma¹, *Aspergillus niger* lipase (ANL, 35 KDa), and *Pseudomonas sp.* lipase (PL1, 30 KDa) were derived from Finnfeeds International² and *Chromobacterium viscosum* lipase (CVL, 73.5 KDa), and *Pseudomonas sp.* lipase (PL2) from Karlan³.

3.3.1 Measurement of lipase activity

Lipase activity was measured in 10 ml of reaction mixture containing 1 mM tris, 2 mM CaCl₂, 150 mM NaCl (Borgstrom and Erlanson-Albertson, 1973). The tributyrin (ICN⁴) concentration and the pH of the mixture were adjusted as required for the particular experiment. The reaction was initiated by addition of about 50 units of lipase activity and the rate of tributyrin hydrolysis monitored by continuously measuring the volume of a stock solution of NaOH added to the mixture to maintain pH over a 6 min period. Initial rates of tributyrin hydrolysis were calculated as the slope of the regression line for the linear portion of the reaction and expressed as micromol (μmol) fatty acids released per minute. Duodenal pH and the temperature of most birds are close to 7 and 40 C,

¹ Sigma, St. Louis, MO 63178-9916 USA..

² Finnfeeds International Inc., Marlborough, SN8 1AA UK.

³ Karlan Research Corporation, Santa Rosa, CA 95403 USA.

⁴ ICN Pharmaceutical Inc., Costa Mesa, CA 92626 USA.

respectively (Duke, 1986; Whitto, 1986). These conditions, in the absence of any pre-incubation states, were chosen as the control. The enzymes were exposed to 40 C for 30 minutes and then activity was measured at pH 7. Lipases were exposed to pH 3 for 30 min at 40 C and then activities were measured at pH 7 to see the effect of acidity on lipases. HCl and/or NaOH were used to adjust the incubation media to the desired pH. In order to study the effect of pepsin (Sigma²), lipases were exposed to 5, 50 and 500 units pepsin per ml of solution for 30 min at pH 3, 40 C; activities were measured at pH 7. Each assay was replicated three times.

Protein concentration of lipases was measured spectrophotometrically at 595 nm (nanometer) using Sigma² procedure 610-A.

Data were analyzed according to the general linear model (GLM) procedure of SAS[®] (SAS Institute, 1985) as a completely randomized design. When treatment effects were significant ($P < 0.05$), Duncan's multiple range test (Steel and Torrie, 1980) was used to compare means.

3.4 Results

3.4.1 Initial rate of tributyrin hydrolysis

Figure 3.1 shows the time course of hydrolysis for two concentrations of tributyrin (0.025 and 1 ml) at 40 C, pH 8 and 7. At both pH conditions, the time course was linear for 4 minutes from initiation. In all subsequent experiments the

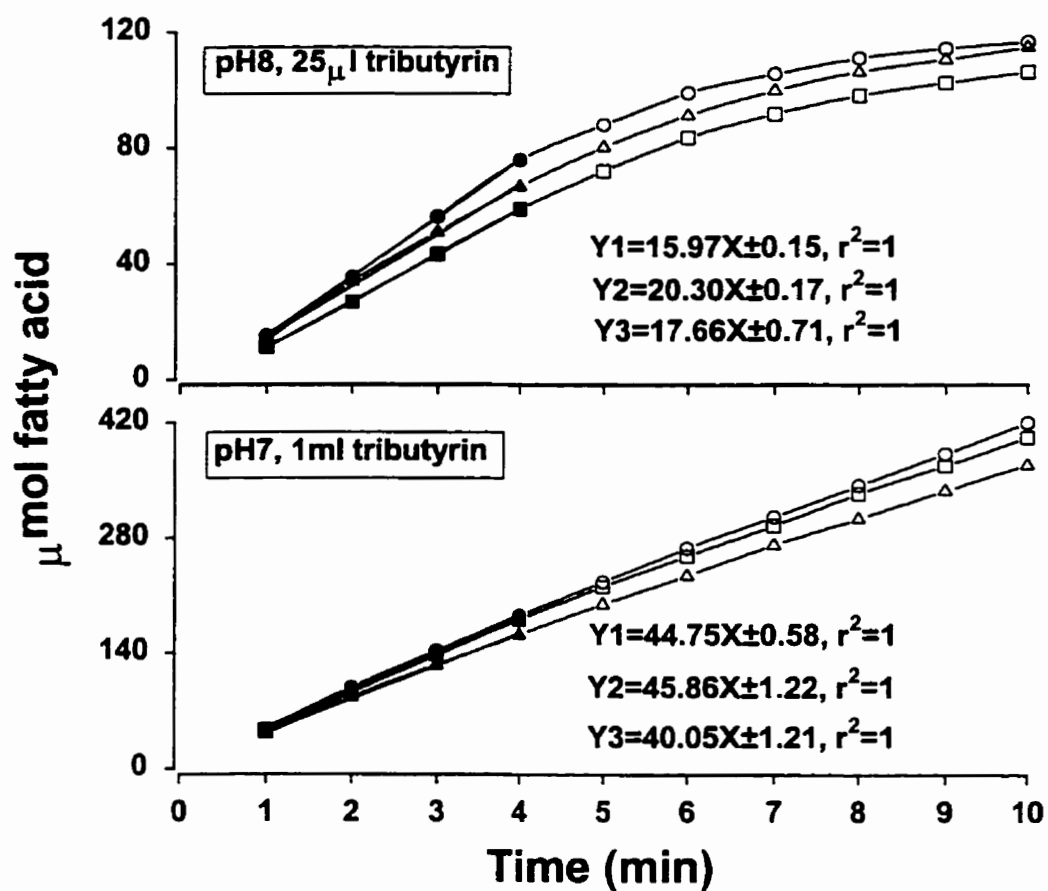


Figure 3.1. Activity of *Pseudomonas sp.* lipase (PL1) at 40 C and ~ 50 units lipase. Y1 to Y3 are the regression equations of replicates of each treatment. The slope of the equations represents the lipase activity.

initial rate of hydrolysis was defined as the slope of linear portion of time course of the reaction.

3.4.2 Kinetics of *Pseudomonas* and crude porcine lipase at pH 7 and 8

Figure 3.2 shows the effect of various concentrations of tributyrin on the initial rate of the reaction in the presence of PL1 and CPL at pH 7 and 8. In all cases a model of a Michaelis-Menten type of enzymatic activity converged to the data. Increasing media pH from 7 to 8 had no effect on maximum velocity (V_{\max}) but was associated with a slight decrease in the affinity of the substrate (K_m) for CPL. For both lipases near saturation of enzyme activity occurred at a substrate concentration of 2 ml. In all further experiments 2 ml of tributyrin was used to approximate the maximum activity of the lipases.

3.4.3 Effect of pH on lipase activity

Activity of lipases at 40 C and different pHs in the absence of any pre-incubation period are presented in Figure 3.3. Enzyme activity tended to increase with higher pH. Very low or no lipase activity was seen for all sources at pH 3 and 4. Maximum activity was seen in the neutral pH range (6 to 8) for all sources except ANL which demonstrated maximum activity at pH 5. Unlike other sources with maximum activity at neutral pH, the activity of RAL was substantially lower at pH 8 than pH 7.

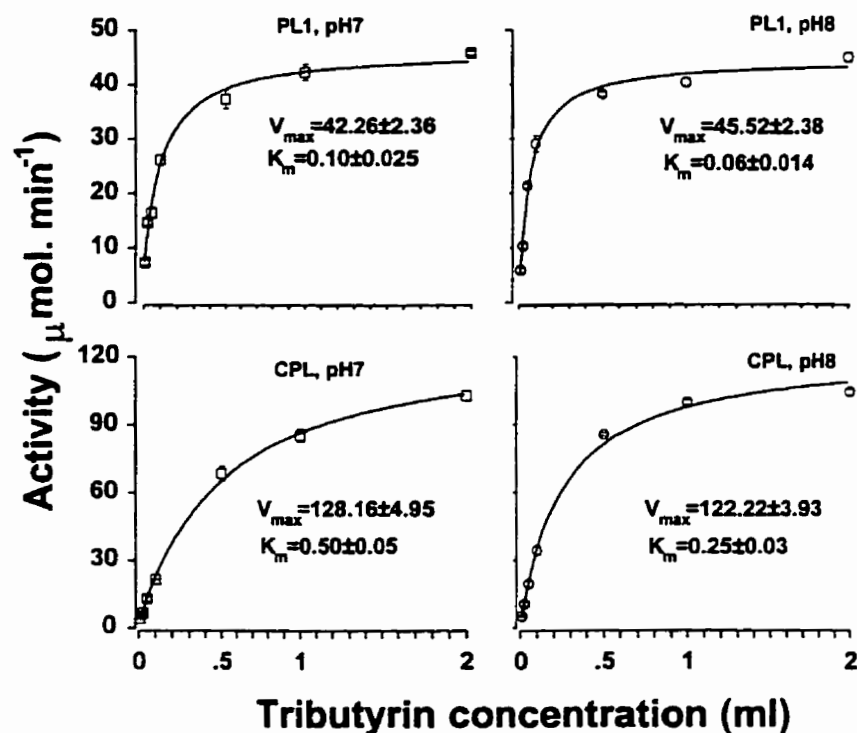


Figure 3.2. Activity of *Pseudomonas sp.* lipase (PL1) and crude porcine lipase (CPL) at pH 7 (\square) and pH 8 (\bullet) and different tributyrin concentrations. Assays were performed at 40 C, and ~ 50 units of lipase. V_{\max} is the maximum velocity obtained under conditions of enzyme saturation and specified pH. K_m is the substrate concentration at which the enzyme has half-maximal velocity.

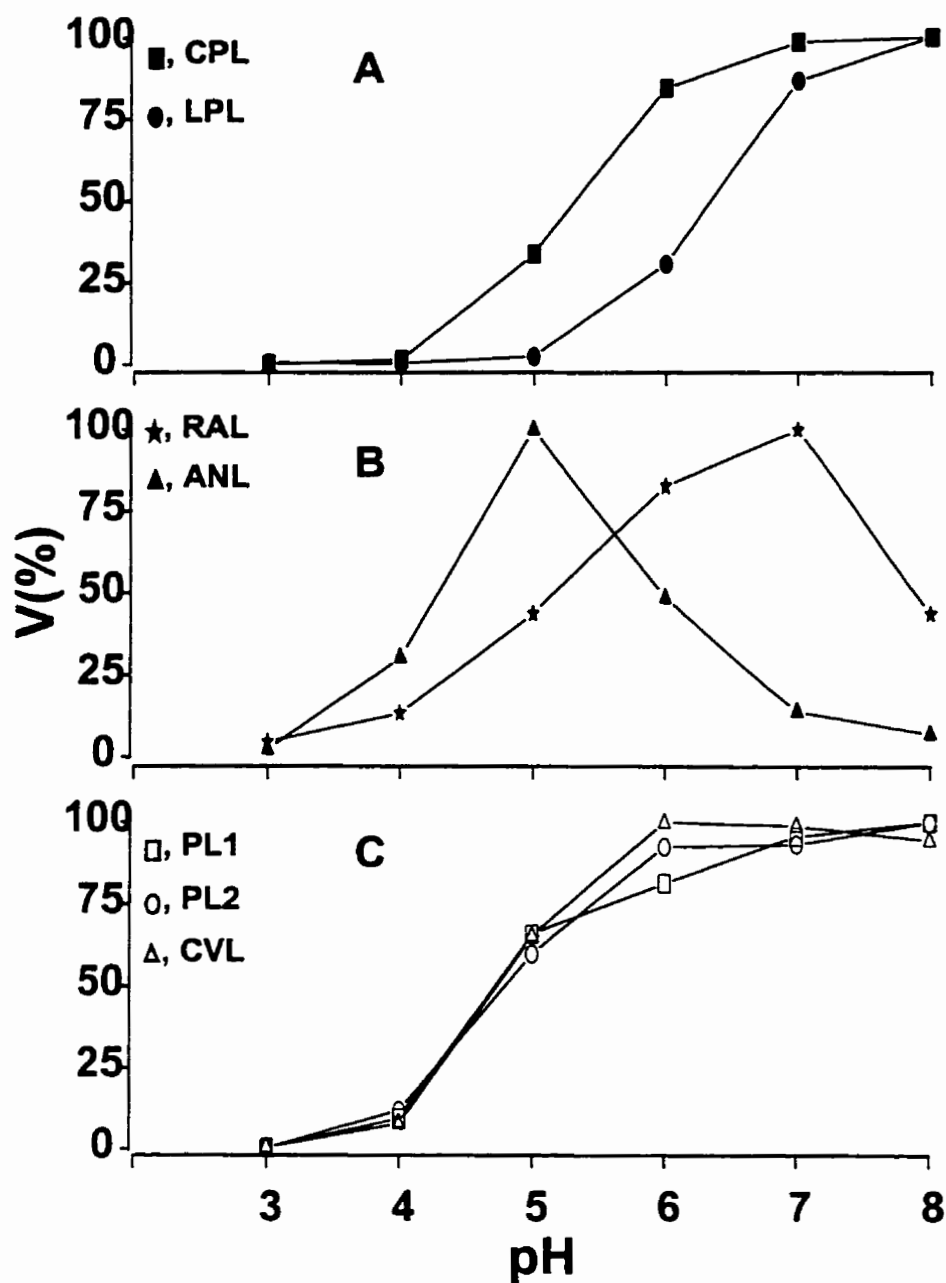


Figure 3.3. Activity of mammalian (A), fungal (B) and bacterial (C) lipases at varying pHs, 40 C and 2 ml tributyrin. V is the rate of hydrolysis in μmol fatty acid released per min as a percentage of those obtained at maximum activity. CPL, crude porcine lipase; LPL, lyophilized porcine lipase; ANL, *Aspergillus niger* lipase; RAL, *Rhizopus arrhizus* lipase; PL1 and PL2, *Pseudomonas sp.* lipase sources; CVL, *Chromobacterium viscosum* lipase.

3.4.4 Effect of pre-incubation conditions on lipase activities

The results of pre-incubation of lipases under varying conditions are shown in Figure 3.4. Pre-incubation for 30 min at 40 C and pH 7, reduced the activity of all lipases except ANL. Longer term exposure to 40 C resulted in a 2.5 fold increase in the activity of ANL measured at pH 7. Pre-incubation of lipases for 30 min at 40 C and in acidic condition (pH 3), markedly inhibited LPL and CPL activity, and reduced the activity (pH 7) of RAL, PL1, PL2, ANL and CVL to 84, 66, 66, 64 and 51% of the control, respectively ($P < 0.05$). Pre-incubation with 500 U/ml pepsin for 30 min at 40 C and pH 3, caused a slight but significant additional inhibition of CVL, PL1 and PL2 activities measured at pH 7. Pre incubation of LPL, CPL, RAL and ANL with pepsin had no effect on their activities.

3.5 Discussion

The titrimetric methodology employed in this study provided an accurate measure of true initial rates of tributyrin hydrolysis and thus it was possible to test kinetic models for fit to initial rate data obtained with varying substrate concentrations. For each of the lipases tested, a model of a Michaelis-Menten type of enzyme activity converged to the data. Maximum velocities were obtained at substrate concentrations greater than 0.5 ml and thus in subsequent experiments initial rates of the reaction were measured with 2 ml of tributyrin incorporated in the mixture to insure that the reaction was measured at maximal velocity.

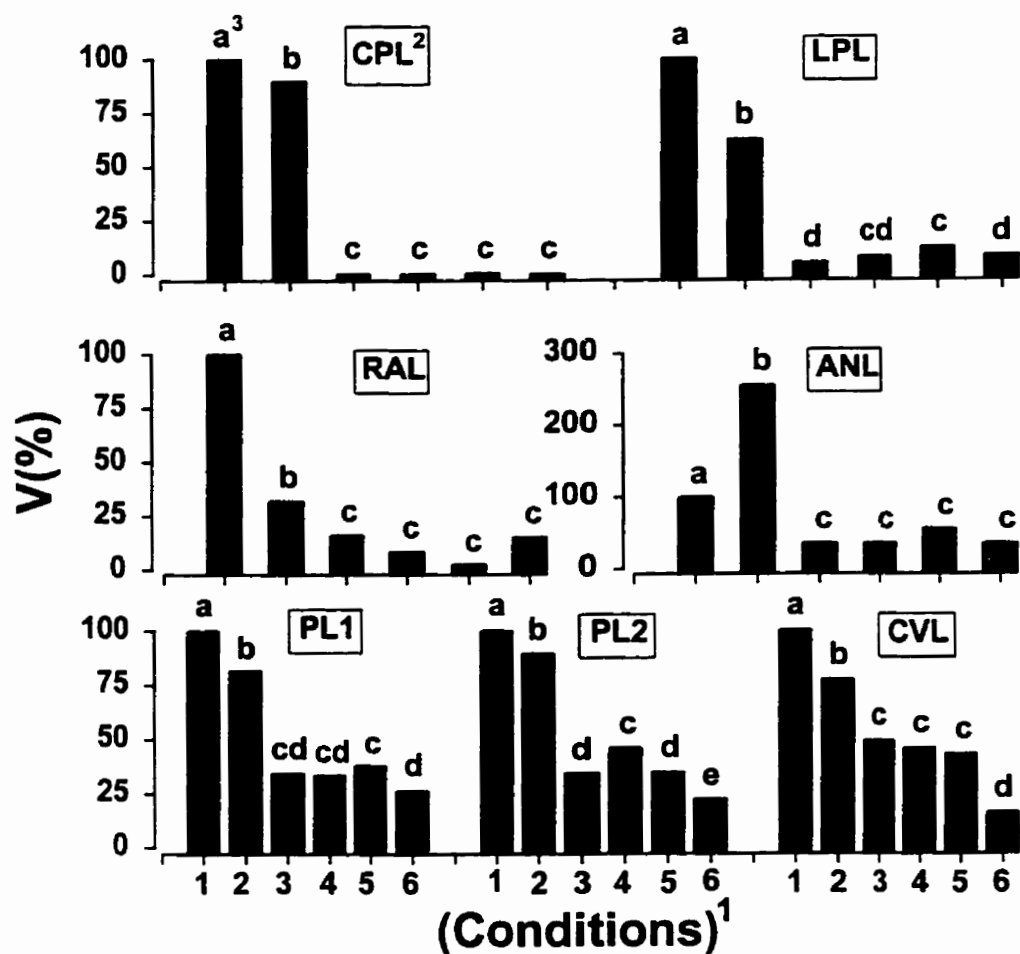


Figure 3.4. Activity of mammalian (top), fungal (middle) and bacterial (bottom) lipases under varying conditions, pH 7, 40 C and 2 ml tributyrin. V is the rate of tributyrin hydrolysis in μmol fatty acid released per minute as a percentage of those obtained at maximum velocity.

¹1) pH 7, 40 C (control); 2) 30 min pre-incubation at 40 C; 3) 30 min pre-incubation at 40 C, pH 3; 4) 30 min pre-incubation at 40 C, pH 3, + 5 U/ml pepsin; 5) 30 min pre-incubation at 40 C, pH 3, + 50 U/ml pepsin; 6) 30 min pre-incubation at 40 C, pH 3, + 500 U/ml pepsin.

²CPL, crude porcine lipase; LPL, lyophilized porcine lipase; ANL, *Aspergillus niger* lipase; RAL, *Rhizopus arrhizus* lipase; PL1 and PL2, *Pseudomonas sp.* lipase sources; CVL, *Chromobacterium viscosum* lipase.

³Bars for each lipase with different letters are significantly different ($P < 0.05$).

A measure of reaction rates at a high concentration of substrate provides a good indication of V_{\max} that is independent of the physical degree of dispersion of the solution. Borgstrom and Erlanson-Albertson (1973) observed that when the substrate was dispersed by sonication, the apparent K_m was drastically decreased. They showed that an increasing amount of substrate concentration has the same effect as sonication of the substrate emulsion. Thus using a high concentration of tributyrin allows the reaction to proceed at its maximal rate. This optimization assures a reliable titrimetric assay that follows the classical Michaelis kinetics.

The lipases tested varied in optimal pH, ranging from 5 to 8. Microbial lipases had higher activities at lower pHs. The results are not in strict accord with those of Zentler-Munro *et al.* (1992). They showed a wide pH optimum range of 2.5 to 5.5 for ANL while our results showed a distinctive peak at pH 5. The results do agree with Stead (1986) who reported that the pH and temperature optimum of many strains of *Pseudomonas* lipase are in the range of 7 to 9 and 30 to 50 C respectively.

Domestic chickens have an internal body temperature of approximately 40 C (Whittow, 1986), and feed passes through the low pH of proventriculus (Winger *et al.*, 1962) in about 30 minutes (Noy and Sklan, 1995) before reaching the neutral pH of the small intestine (Duke, 1986). Exposure of lipases to 40 C, for 30 minutes decreased their activities as expected but ANL showed the opposite

response increasing 2.5 fold in activity. This result was unexpected and requires further investigation.

Pre-incubation of CPL and LPL at pH 3 for 30 minutes (40 C) inhibited their activities when measured at pH 7. This confirms that porcine lipase is not acid resistant and inactivation of porcine or other mammalian pancreatic lipases before they reach the small intestine can contribute to the failure of oral enzyme replacement therapy for treatment of fat malabsorption (steatorrhoea) in human studies (DiMagno *et al.*, 1977). To protect pancreatic lipase from acid denaturation, antacids, inhibitors of gastric acid secretion (Graham, 1982) or enteric coated lipase (Roberts, 1989) have been used. However these methods are likely not applicable in the poultry industry because of the grinding activity of the gizzard and the lower retention time of feed in the duodenum. Enzyme replacement therapy with acid-stable lipases may be more appropriate in birds. Microbial lipases were more resistant to acidic conditions and it is probable that these enzymes should work better than pancreatic lipases.

Pre-incubation with pepsin under acid conditions that approximate the stomach is known to have variable effects on enzyme activity dependent upon the enzyme in question. Heizer *et al.* (1965) showed that trypsin was inactivated by pepsin and acid while pancreatic lipase was inactivated by a pH of less than 4 in human subjects. DiMagno *et al.* (1977) also showed that trypsin and pancreatic lipase can be inactivated by pepsin and acid of the stomach, respectively. Zentler-

Munro *et al.* (1992) showed that ANL is pepsin and trypsin resistant and any inhibition below pH 4 is completely reversible. In our study, incorporation of pepsin in the pH 3 pre-incubation media had no further inhibitory effect on the acid-sensitive lipase, CPL, LPL and ANL. The activity of ANL and PL1 was not affected by pepsin addition to the acid pre-incubation media. At the highest concentration of pepsin the activity of PL2 and CVL was partially inhibited relative to the acid only pre-incubation treatment. Despite this loss, these sources still retained 16-27% of their maximal activity. Our findings are in agreement with Raimondo and DiMagno (1994) in that bacterial lipase lipolytic activity was shown to be more resistant to inactivation by acid than the lipolytic activity of porcine lipase. As such one would predict that bacterial lipases would retain more activity during passage to the small intestine than the lipolytic activity of either human or fungal lipase.

3.6 Conclusions

In summary, not all lipases retain activity during incubation in conditions that mimic the chicken proventriculus. The activity of CPL, LPL and RAL was very low after low pH exposure and therefore these lipases are not considered suitable candidates for use in diet to prevent steatorrhoea in young chickens. Despite some loss of activity, PL1, PL2, CVL and ANL may be suitable sources of

lipase for dietary supplementation. Further research is required to establish whether these enzymes can also be active in the conditions of the small intestine.

4.0 STABILITY OF PORCINE AND MICROBIAL LIPASES TO CONDITIONS WHICH APPROXIMATE THE SMALL INTESTINE OF THE YOUNG BIRDS

4.1 Abstract

In vitro experiments were conducted to determine the stability of lipase activities from bacterial, fungal and animal sources under conditions that approximate those of digesta in the small intestine. In the first experiment, the effects of pre-incubation with trypsin (TR; 500, 1000, and 2000 U/ml), chymotrypsin (CTR; 200, 400, and 800 U/ml), and trypsin plus chymotrypsin (TC; 2000 U/ml TR + 800 U/ml CTR) for 30 min at 40 C on lipase activities from 2 sources of *Pseudomonas sp.*(PL1, PL2), *Chromobacterium viscosum* (CVL), and *Aspergillus niger* (ANL) were determined. None of the enzymes were inhibited by TR. CTR decreased the activity of all of the lipases. TC had no additional effect on the activities of PL1 and PL2; however, ANL and CVL activities were further decreased relative to the CTR only treatment. In the second study, the effects of Na taurodeoxycholate (NaTDC; 0.1-16 mM) on the activities of PL1, PL2, CVL, ANL and crude porcine lipase (CPL) at 23 and 40 C were evaluated. At 23 C, in order of potency, NaTDC inhibited the activities of ANL, CPL and CVL. At this temperature, NaTDC did not inhibit PL1 and PL2. An increase in the temperature to 40 C increased the activity of all of the enzymes tested. At 40 C, NaTDC had similar effects on lipase activities as seen at 23 C, however, higher NaTDC levels

were required to inhibit ANL activity, and only a partial inhibition of CPL occurred. At 23 C, porcine colipase (PCL) restored the activity of CPL but had no effect on ANL and CVL in the presence of inhibitory levels of NaTDC. At 40 C, PCL had no effect on NaTDC inhibition of lipase activity. The results of this study indicate that lipase from *Pseudomonas sp.* is more stable than *Chromobacterium viscosum* and *Aspergillus niger* lipases, and colipase addition has no beneficial effects on microbial lipase activities under conditions that approximate those in the small intestine.

4.2 Introduction

An inadequacy in pancreatic lipase secretion may contribute to the poor digestibility of fat in comparison to fat digestion in older birds with a fully developed digestive function (Sell *et al.*, 1986). Dietary supplementation with sources of lipases has the potential to improve the efficiency of triglyceride hydrolysis and thus promote fat digestion in the young bird.

If supplementation with sources of lipases are to be effective, then the lipase must survive conditions of passage through the proventriculus and gizzard and retain hydrolytic activity in the digesta of the small intestine. In the previous paper, (Chapter 3) the stability of bacterial (*Pseudomonas sp.*, PL1 and PL2, and *Chromobacterium viscosum*, CVL), fungal (*Rhizopus arrhizus*, RAL, and *Aspergillus niger*, ANL) and animal (crude porcine, CPL) sources of lipases were

tested under conditions that approximate the proventriculus. The bacterial lipases (PL1, PL2 and CVL) and one source of fungal lipase (ANL) were found to be relatively stable under the acidic and enzyme conditions that approximate the proventriculus when compared to the stability of the animal lipase and RAL.

After passage through the proventriculus and gizzard, dietary lipase must function to hydrolyze triglycerides in the small intestine. High concentrations of bile salts contribute to fat emulsification and are a characteristic of the digesta in the small intestine. However hydrolysis of emulsified triglycerides by pancreatic lipase can be inhibited by bile salts that displace lipase from the lipid-water interface and make it inactive. This inhibition can be reversed by another pancreatic protein, colipase. Colipase acts as a specific cofactor to pancreatic lipase and enables the latter enzyme to adsorb at the oil-water interface (Borgstrom *et al.*, 1979a, b). Thus colipase is essential for full activity of lipase in the gut lumen (Erlanson-Albertson, 1992a, b). Colipase is produced in the pancreas of chickens (Bosc-Bierne *et al.*, 1984) and is similar to colipase from mammalian species (Canioni *et al.*, 1975; Rinderknecht, 1986). It is secreted as pro-colipase and converted to its physiologically active form, colipase, by trypsin (Borgstrom *et al.*, 1979b; Erlanson-Albertson, 1992a).

Although the presence of colipase has been established in poultry species, little information is available on the factors that affect its secretion and its relation to the pancreatic lipase. Studies on swine and rats indicate a lack of co-ordination

between lipase and colipase secretion when high levels of fat are fed (Mouroto and Corring, 1979; Ouagued *et al.*, 1980). A high fat diet in swine causes a greater elevation of lipase than colipase (80% vs 37%). It is suggested that when rats are fed a high fat diet, colipase is secreted into the small intestine faster than lipase (Ouagued *et al.*, 1980). In turn, this can result in a depletion of pancreatic colipase. The possible lack of co-ordination of lipase and colipase secretion, along with the lipase deficiency may contribute to the poor utilization of fats by young birds.

The objective of this study was to evaluate the activity of bacterial, fungal and animal sources of lipase under conditions of temperature, bile salt, colipase and proteolytic enzymes that approximate environments within the lumen of the small intestine.

4.3 Materials and Methods

Crude porcine lipase (CPL, 50 KDa), was obtained from Sigma¹ while *Aspergillus niger* lipase (ANL, 35 KDa) and one *Pseudomonas sp.* lipase source (PL1) were a gift from Finnfeeds International² *Chromobacterium viscosum* lipase (CVL, 73.5 KDa) and another *Pseudomonas sp.* lipase source (PL2) were purchased from Karlan³. Trypsin (TR), chymotrypsin (CTR), Na cholate:Na

¹ Sigma, St. Louis, MO 63178 USA.

² Finnfeeds International Inc., Marlborough, Wiltshire, SN8 1AA UK.

³ Karlan Research Corporation, Santa Rosa, CA 95403 USA.

deoxycholate (NaDC) and Na taurodeoxycholate (NaTDC) were also obtained from Sigma¹. Porcine colipase (PCL) was purchased from Calzyme⁴

4.3.1 Measurement of lipase activity

Previous work in our lab (Chapter 3) established that the maximum velocity of tributyrin⁵ hydrolysis occurs when 2 ml of tributyrin are mechanically emulsified at high speed into 10 ml of a reaction mixture containing 1 mM tris-HCl buffer (pH 7), 2 mM CaCl₂ and 150 mM NaCl as described previously (Chapter 3). Initial rates of tributyrin hydrolysis were measured titrimetrically by the quantity of NaOH added per unit of time required to maintain a constant pH of 7 and calculated as the slope of the regression line for the linear portion of the reaction. The reaction rates are expressed as a percentage of the rate obtained under control conditions (pH 7 and 40 C). The lipases were exposed to a) TR (500, 1000 and 2000 U/ml), b) CTR (200, 400 and 800 U/ml), c) 2000 U/ml TR + 800 U/ml CTR, and d) NaCD (0.5, 1, 2, 4, 8 and 16 mM). The lipases also exposed to NaTDC (0.1, 0.25, 0.5, 1, 2, 4, 8 and 16 mM) at 23 and 40 C. The lipases which were inhibited by bile salts were exposed to PCL. PCL was added at 5 fold excess relative to the concentration of lipase.

Protein concentration of lipases was measured spectrophotometrically at 595 nm (nanometer) using Sigma¹ procedure 610-A.

⁴ Calzyme Laboratories Inc., San Luis Obispo, CA 93401 USA.

⁵ ICN Pharmaceutical Inc., Costa Mesa, CA 92626 USA.

4.3.2 Statistical analysis

Data were analyzed according to the general linear model (GLM) procedure of SAS[®] (SAS Institute, 1985) as a completely randomized design. When treatment effects were significant ($P < 0.05$), Duncan's multiple range test (Steel and Torrie, 1980) was used to compare means.

4.4 Results

The effects of pre-incubation at 40 C and pH 7 for 30 minutes with or without TR on the activity of lipases when measured at 40 C and pH 7 are shown in Figure 4.1. Pre-incubation for 30 minutes at 40 C and pH 7, reduced the activity of all lipases except ANL. Pre-incubation for 30 min at 40 C resulted in a 2.5 fold increase in the activity of ANL. None of the enzymes were inhibited by TR. Pre-incubation for 30 minutes with 500 U/ml TR at 40 C caused 1.7 fold increase in ANL activity compared to the activity obtained after 30 minutes pre-incubation with no enzyme. At 1000 and 2000 U/ml of TR lipase activities were not different than that obtained after pre-incubation in the absence of enzyme.

The effects of pre-incubation at 40 C and pH 7 for 30 minutes with or without CTR, or TR plus CTR, on the activity of lipases measured at 40 C and pH 7 are shown in Figure 4.2. The activity of all lipases was affected by CTR compared to the activity obtained after 30 minutes pre-incubation with no enzyme. The activity of all lipases except ANL decreased in response to higher

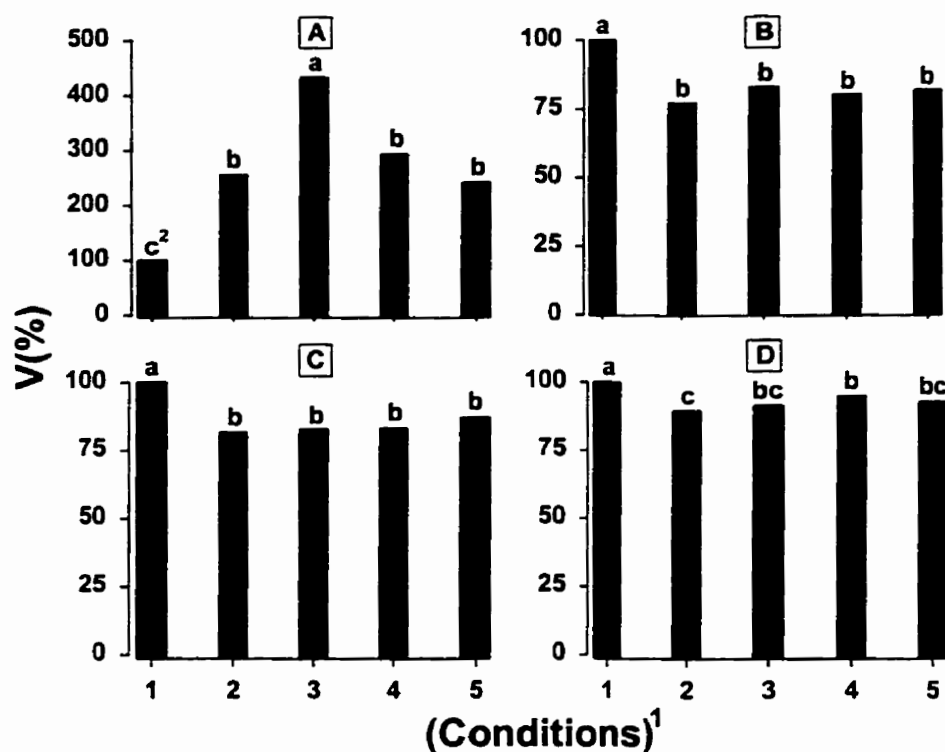


Figure 4.1. The effect of pre-incubation at 40 C for 30 minutes with and without trypsin on the activity of *Aspergillus niger* lipase (A), *Chromobacterium viscosum* lipase (B), *Pseudomonas sp.* PL1 (C) and *Pseudomonas sp.* PL2 (D) lipase sources at pH 7 and 40 C using tributyrin as substrate. V is the rate of tributyrin hydrolysis in μmol fatty acid released per minute as a percentage of that obtained in the absence of any pre-incubation conditions.

¹1) pH 7, 40 C (control); 2) pH 7, 30 min. at 40 C; 3) pH 7, 30 min. at 40 C, and 500 U/ml trypsin; 4) pH 7, 30 min. at 40 C and 1000 U/ml trypsin; 5) pH 7, 30 min. at 40 C and 2000 U/ml trypsin.

²Values for each lipase with different letters are significantly different ($P < 0.05$).

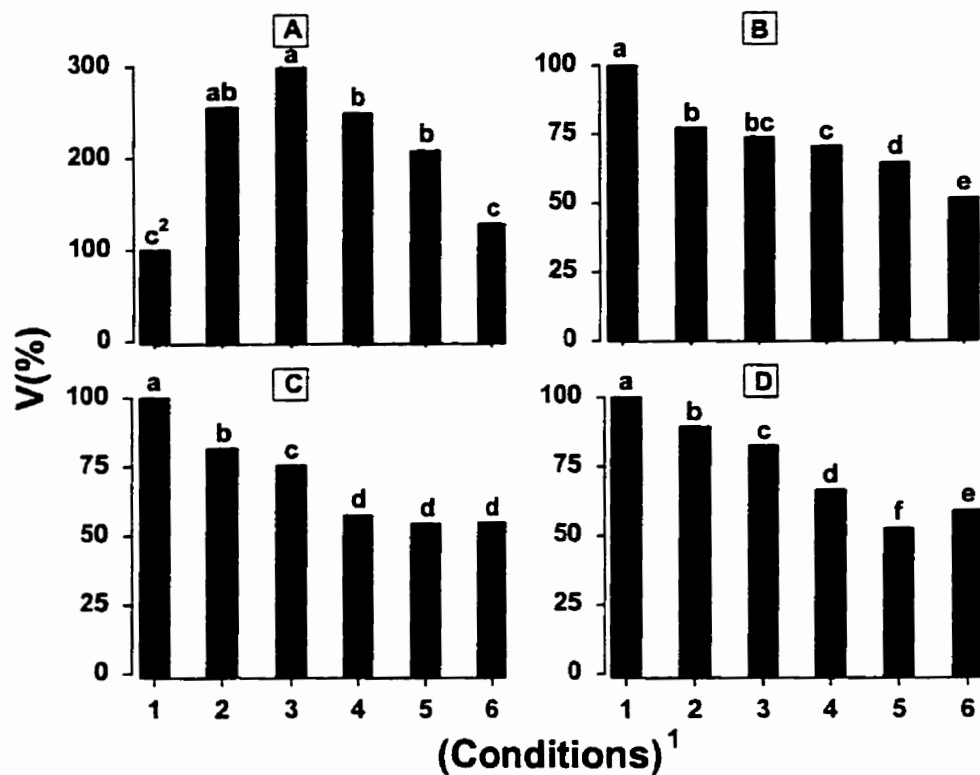


Figure 4.2. The effect of chymotrypsin on lipase activity after various pre-incubation conditions. Activity of *Aspergillus niger* lipase (A), *Chromobacterium viscosum* lipase (B), *Pseudomonas sp.* PL1 (C) and *Pseudomonas sp.* PL2 (D) lipase sources at pH 7, and 40 C using tributyrin as the substrate. V is the rate of tributyrin hydrolysis in μmol fatty acid released per minute as a percentage of those obtained in the absence of any pre-incubation conditions.

¹1) pH 7, 40 C (control); 2) pH 7, 30 min. at 40 C; 3) pH 7, 30 min. at 40 C and 200 U/ml chymotrypsin; 4) pH 7, 30 min. at 40 C and 400 U/ml chymotrypsin; 5) pH 7, 30 min. at 40 C and 800 U/ml chymotrypsin; 6) pH 7, 30 min. at 40 C and 2000 U/ml trypsin + 800 U/ml chymotrypsin.

²Values for each lipase with different letters are significantly different ($P < 0.05$).

concentrations of CTR. The activity of ANL increased at the lowest concentration of CTR but then decreased at higher levels of CTR. TC had no additional effect on the activities of PL1 and PL2; however, ANL and CVL activities were decreased relative to the CTR only treatment.

The effects of NaCD on lipase activities are presented in Figure 4.3. Compared to control conditions, NaCD had only a minor effect on PL1 and PL2 activities, however, the activities of CVL and ANL were decreased. By increasing the concentration of NaCD to 16 mM, a gradual decrease was observed in the activity of both enzymes. At 16 mM, NaCD caused a 25% inhibition of CVL activity while ANL activity decreased by 70%.

The effects of varying concentrations of NaTDC on lipases at 23 and 40 C are shown in Table 4.1. In descending order of potency, NaTDC inhibited the activities of ANL, CPL and CVL at both temperatures. At these temperatures, NaTDC did not inhibit PL1 and PL2. At low concentrations of NaTDC, the activities of all three bacterial lipases tended to increase and this increase was greater for PL1 and PL2 sources than CVL. ANL was very sensitive to even low concentrations of NaTDC and complete inhibition was seen at 0.25 mM. An increase in the temperature from 23 to 40 C increased the activity of all lipases tested (Table 4.1). At 40 C, NaTDC had similar effects on lipase activities, however, higher NaTDC levels were required to inhibit ANL activity, and only a partial inhibition of CPL occurred.

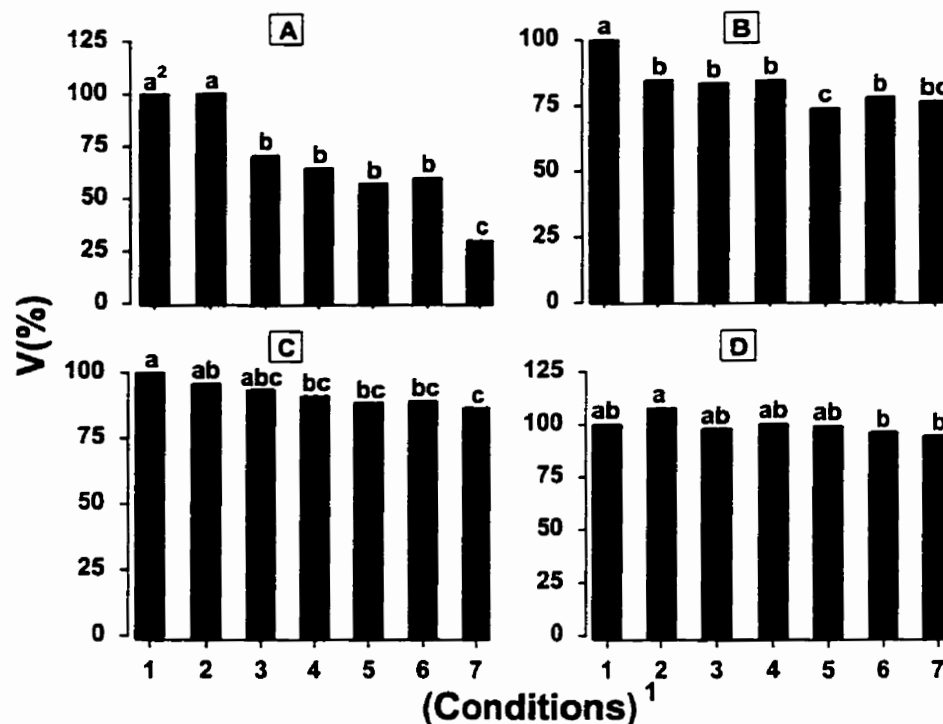


Figure 4.3. The effect of varying concentrations of a 1:1 mixture of Na-cholate:Na-deoxycholate (NaCD) on the activity of *Aspergillus niger* (A), *Chromobacterium viscosum* (B), *Pseudomonas sp.* PL1 (C) and *Pseudomonas sp.* PL2 (D) lipase sources measured at pH 7 and 40 C using tributyrin as the substrate. V is the rate of tributyrin hydrolysis in μmol fatty acid released per minute as a percentage of those obtained in the absence of NaCD.

¹1) no added NaCD; 2) 0.5 mM NaCD; 3) 1 mM NaCD; 4) 2 mM NaCD; 5) 4 mM NaCD; 6) 8 mM NaCD; 7) 16 mM NaCD

²Values for each lipase with different letters are significantly different ($P < 0.05$).

TABLE 4.1. The effects of sodium taurodeoxycholate on the activity of lipases at 23 and 40 C (micromol*1000/min.)

	ANL ²		PL1		PL2		CVL		CPL	
NaTDC (mM)	23 C	40 C	23 C	40 C	23 C	40 C	23 C	40 C	23 C	40 C
0 (control)	353.0 ^{a3}	489.7 ^a	232.3	577.3	408.7 ^c	1260.7	137.3 ^{ab}	359.0 ^a	83.0 ^a	179.7 ^a
0.1	107.7 ^b	352.0 ^b								
0.25	0 ^c	72.0 ^c								
0.5	0 ^c	44.7 ^{cd}								
1	0 ^c	0 ^d	272.0	585.3	533.0 ^b	1276.3	158.3 ^a	394.3 ^a	28.7 ^b	208.7 ^a
2			253.7	557.7	693.7 ^a	1190.3	128.7 ^{ab}	367.7 ^a	6.7 ^{bc}	165.3 ^a
4			231.7	564.7	584.0 ^b	1184.7	117.3 ^{bc}	356.7 ^a	0 ^c	96.3 ^b
8			235.7	579.7	582.0 ^b	1129.7	89.0 ^c	288.7 ^{ab}	0 ^c	91.7 ^b
16			245.7	578.0	584.0 ^b	1123.0	45.0 ^d	232.3 ^b	0 ^c	90.3 ^b
SEM	7.67	17.08	23.59	64.31	26.87	57.41	9.99	32.36	7.43	19.04
P value	0.0001	0.0001	0.811	0.999	0.0003	0.348	0.0001	0.035	0.0001	0.002

¹Lipase activity measured at pH 7 using tributyrin as substrate.

²ANL, *Aspergillus niger* lipase; PL1 and PL2, *Pseudomonas sp.* lipases; CVL, *Chromobacterium viscosum* lipase; CPL, crude porcine lipase; NaTDC, sodium taurodeoxycholate.

³Means in columns with the same superscripts are not significantly different.

At 23 C, porcine colipase (PCL) restored the activity of CPL but had no effect on ANL and CVL in the presence of inhibitory levels of NaTDC (Figure 4.4). At 40 C, PCL had no effects on NaTDC inhibition of lipase activity.

4.5 Discussion

In this study, different sources of lipase were evaluated under conditions that approximate the small intestine of young birds. Birds have an internal body temperature of about 40 C (Whittow, 1986). Feed passes through the low pH of the proventriculus (Winger *et al.*, 1962) and reaches the nearly neutral pH of the duodenum (Duke, 1986) where it is exposed to pancreatic secretions and bile salts. The effects of TR and CTR on the activity of ANL and CVL observed in this study are similar to the effect of TR and CTR on pancreatic lipase observed in human studies (Layer *et al.*, 1986; Thiruvengadam and DiMagno, 1988). In duodenal contents, human lipase loses 76% of its activity after 2 hours when incubated at 37 C (Thiruvengadam and DiMagno, 1988). In the present study CTR was a more potent inhibitor of pancreatic lipase relative to TR. The authors concluded that pancreatic lipase is relatively more resistant to TR digestion and that TR digestion of lipase requires the presence of CTR. The inhibitory effect of CTR was seen in all lipases tested in our study. The combination of TR with CTR showed an additional effect on ANL and CVL but had no more effect on *Pseudomonas sp.* lipase sources, PL1 and PL2. In our *in vitro* study bacterial lipase originating from

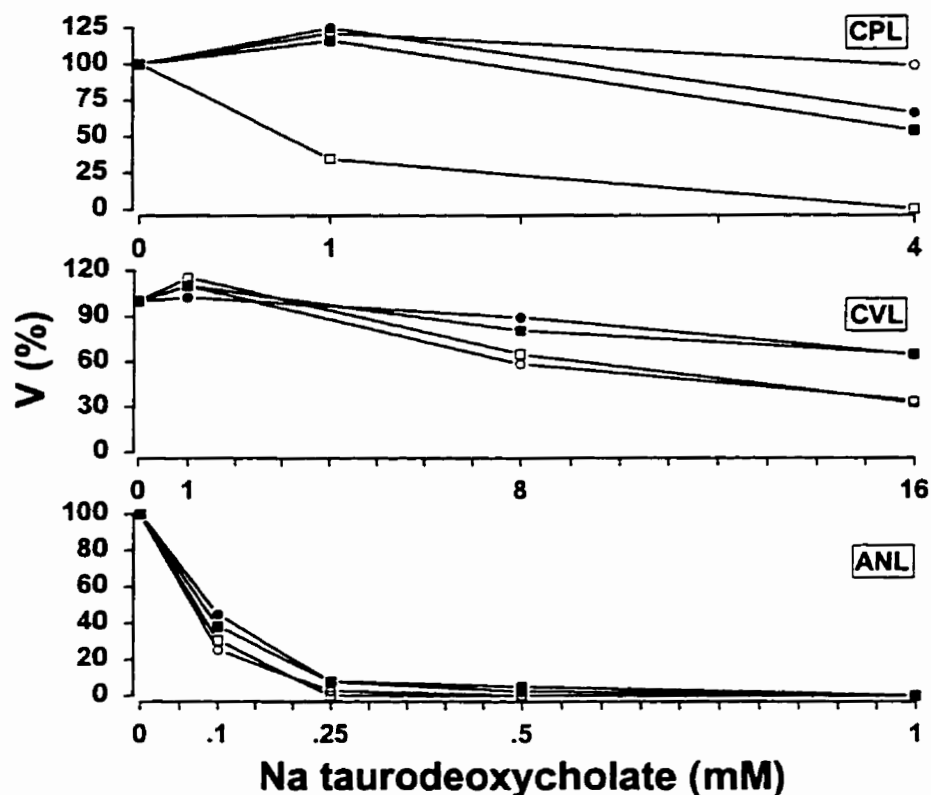


Figure 4.4. The effects of Na taurodeoxycholate on the activity of crude porcine lipase (CPL), *Chromobacterium viscosum* lipase (CVL) and *Aspergillus niger* lipase (ANL) at pH 7, 23 (open symbols) or 40 C (solid symbols) , with (●, ○) or without (■, □) the addition of porcine colipase (PCL). PCL was added at 5 fold excess relative to the concentration of lipase. In this experiment 0.5 ml of tributyrin was incorporated into the reaction mixture. V is the rate of hydrolysis in μmol fatty acid released per minute as a percentage of those obtained in the absence of Na-taurodeoxycholate and colipase.

Pseudomonas sp. was more resistant to the inhibitory effect of TC than CVL and the fungal lipase, ANL.

The effect of conjugated bile salts on the activity of pancreatic lipase has been shown to be concentration dependent (Borgstrom and Erlanson-Albertson, 1973). Below the critical micellar concentration (CMC) conjugated bile salts slightly stimulate the initial rate of hydrolysis as seen in this study with NaCD and *Pseudomonas* lipase and with NaTDC and PL1, PL2, CVL, and CPL. It is reported that, at pH 8 and 25 C, concentrations of Na cholate, Na deoxycholate, and NaTDC lower than the CMC, stimulate tributyrin hydrolysis by pancreatic lipase activity (Borgstrom and Erlanson-Albertson, 1973). With increasing concentration of Na cholate, over the CMC, there is a gradual decrease in lipase activity to more than 70% inhibition by 8 mM concentration. At this level, about 45% of porcine lipase activity is inhibited by Na deoxycholate. Higher concentrations of both bile salts didn't show more inhibition and the activity of porcine lipase remained constant. The CMC ranges of bile salts are different. It is reported that the CMC ranges of Na cholate, Na deoxycholate, and NaTDC are 2.6 to 3.1, 0.8 to 1.2, and 0.8 to 1.3 mM, respectively (Borgstrom, 1977). Bile salts at low concentrations can stimulate the rate of hydrolysis by increasing the surface area of the substrate (Momsen and Brockman, 1976). Alternately, Schoor and Melius (1970) believe that the stimulatory or inhibitory effect of bile salts is dependent on the thickness of its layer around the substrate. The stimulatory effect

of NaTDC was seen in most bacterial lipases tested in this study above the CMC at both 23 and 40 C. No stimulatory effect of NaTDC on ANL was found and the inhibitory effect was below the CMC. The results for ANL are similar to those obtained from another fungal lipase, *Rhizopus arrhizus* lipase (Semeriva and Dufour, 1972). *Rhizopus arrhizus* lipase was strongly inhibited by bile salts and no stimulatory effect was observed.

In the current study PCL was found to restore CPL activity but have no effect on microbial lipases, ANL and CVL. The results are similar to those obtained by Canioni *et al.* (1977) who showed that *Rhizopus arrhizus* lipase was completely inactivated by NaTDC. Inhibition occurred at bile salt concentrations below the CMC, and colipase had no activating effect either in the absence or in the presence of bile salts. They emphasized that colipase synthesized in the mammalian pancreas displays a specific interaction with lipases of the same origin.

Although pancreatic and microbial lipases (fungal) seem to be equally sensitive to bile salt inhibition, they act differently with respect to their ability to interact with the pancreatic colipase. Colipase behaves as a specific cofactor for mammalian and bird pancreatic lipases but fails to interact with microbial lipases at the substrate/water interface.

Increasing the temperature from 23 to 40 C increased the activity of all lipases tested. It is suggested that higher temperatures decrease surface pressure at the oil/water interface (Canioni *et al.*, 1977) and may increase lipase penetration

into the interface in the absence or presence of bile salts. It seems that a higher proportion of lipase is active at 40 C than at 23 C and consequently causes an increase in the rate of substrate hydrolysis. Colipase had no effect on CPL at 40 C in the presence of NaTDC. It has been suggested that with increasing surface pressure the activation of porcine lipase by colipase increases (Wieloch *et al.*, 1981). This is in agreement with our finding that colipase failed to increase CPL activity in the presence of NaTDC at 40 C whereas it did at room temperature.

4.6 Conclusions

The results of this study indicated that the bacterial *Pseudomonas sp* lipase is more stable than the porcine and fungal lipases tested, and colipase addition had no beneficial effects on microbial lipase activities under conditions that approximate the small intestine. Previously we showed that bacterial lipases are also more resistant to conditions that approximate the proventriculus. Although *Pseudomonas sp* lipase showed more stability in these *in vitro* studies, more *in vivo* research is required to determine whether dietary supplementation of *Pseudomonas* lipase can improve fat utilization in young birds.

5.0 THE EFFECT OF DIETARY SUPPLEMENTATION OF *PSEUDOMONAS SP.* LIPASE ON FAT DIGESTIBILITY IN BROILER CHICKENS

5.1 Abstract

An *in vivo* experiment was conducted to determine the effect of dietary *Pseudomonas sp.* lipase on fat digestibility and performance of broiler chickens. *Pseudomonas sp.* lipase was obtained as a predissolved solution containing 15800 units per ml and added at 0, 50, 100, 200 and 400 units per g of diet to a corn-soybean meal diet containing 8% pure beef tallow. Broilers were randomly distributed to battery cages and fed the experimental diets from 0 to 35 days of age. Intestinal contents were analyzed for lipase activity at 7, 14, 21 and 35 days of age, and fat digestibility was assessed from feces collected at 5 to 7, 12 to 14, 19 to 21 and 33 to 35 days of age. Body weight gain, feed intake and feed efficiency decreased in response to increasing levels of dietary lipase. Absolute pancreatic weight was unaffected by dietary treatment but proportional pancreas weight showed a positive linear relationship to lipase level at 14 and 35 days of age. Dietary enzyme affected intestinal lipase activity with a quadratic increase at 14 days of age and a linear decrease at 21 and 35 days of age. Fat digestibility was negatively affected by lipase addition at 19-21 and 33-35 days of age. The apparent digestibility of individual fatty acids was also affected by dietary enzyme.

Of the major dietary fatty acids, enzyme addition tended to increase the digestibility of C16:0 and 18:0, while decreasing the digestibility of C16:1 and C18:1. The use of 400 units of lipase per g of diet resulted in 83% hydrolysis of triglycerides in the diet during mixing and/or storage of the feed. Dietary *Pseudomonas sp.* lipase decreased performance and failed to increase the overall digestion of fat. It is hypothesized that the complete hydrolysis of triglycerides may be at least partially responsible for these effects.

5.2 Introduction

Fats are useful and widely used ingredients in poultry feeds. However, the ability of birds to digest fats is not universally high. In particular, saturated fats are poorly utilized by young birds (Sell *et al.*, 1986; Leeson and Atteh, 1995). The underdeveloped status of the digestive tract at hatch has been suggested to be the major reason for the poor fat digestibility and research has primarily focused on the role of bile salts and enzyme concentration.

Bile salts and detergents have been added to poultry diets in an attempt to improve fat digestion but the success had been limited (Gomez and Polin, 1976) and the fat digestibility values obtained are still far from the values seen in adult birds. Perhaps this is not surprising since a relatively high concentration of bile salts has been found in the jejunal contents of 2-day old chicks (Green and Kellogg, 1987). In fact the levels were higher than for older birds where fat

digestion was not considered a problem. Therefore, the evidence to date suggests that bile salt insufficiency is not the only or primary cause of poor fat utilization in young birds (Gomez and Polin, 1974, 1976).

A deficiency of lipase has also been suggested to be a factor in the low fat utilization of young birds. It has been uniformly shown that the concentration of digestive enzymes increase with age in poultry (Brannon, 1990; Pubols, 1991). As an example, lipase secretion has been shown to increase 20 fold between 4 and 21 days of age in the chick (Noy and Sklan, 1995). The role of lipase in birds is the same as mammalian species with this enzyme responsible for the hydrolysis of the one and three positions of triglycerides to yield 2-monoglycerides and free fatty acids (Borgstrom *et al.*, 1979a, b; Erlanson-Albertson, 1992a). The activity of lipase is also related to bile salts and colipase. Bile salts are essential for fat emulsification but can also displace lipase from the lipid-water interface and thereby eliminate its activity. This inhibition can be reversed by pancreatic colipase. Colipase acts as a specific co-factor to lipase and enables the latter enzyme to adsorb to the oil-water interface. Thus, colipase is essential for full activity of lipase in the small intestine (Erlanson-Albertson, 1992a, b). Chicken colipase has been isolated (Bosc-Bierne *et al.*, 1984) and as with its mammalian counterpart is converted to its physiological active form by trypsin (Borgstrom *et al.*, 1979a, b; Erlanson-Albertson, 1992a).

Dietary lipase has been considered as a solution for steatorrhoea in several species. Lipase has been used as a dietary supplement in pancreatic insufficiency in humans but with mixed success (DiMagno *et al.*, 1973). Reasons for the failure of dietary lipase to overcome pancreatic insufficiency have been suggested to include destruction of the enzyme in the stomach and/or small intestine, and variability in the potency of commercial lipase supplements (DiMagno *et al.*, 1977; Raimondo and DiMagno, 1994). In poultry, Polin *et al.* (1980) showed that dietary supplementation of crude porcine lipase in a corn-soybean meal based diet containing 4% tallow did not eliminate steatorrhoea in young chicks.

In vitro studies in our laboratory assayed the activity of different lipase sources under conditions that approximate the proventriculus and small intestine of young birds. This research showed that the bacterial *Pseudomonas sp.* lipase was stable (Chapters 3 and 4) to the conditions of the digestive tract and showed some promising characteristics in terms of its use as a dietary supplement. No information is available on the effects of dietary supplementation of bacterial lipases under *in vivo* conditions in young birds and therefore this study focused on the dietary supplementation of *Pseudomonas sp.* lipase in broiler diets containing 8% tallow. The hypothesis is that this lipase will improve fat utilization in young chickens.

5.3 Materials and Methods

The objective of this experiment was to determine the effect of dietary lipase on the performance and digestive characteristics of broiler chicks. An experimental liquid form of *Pseudomonas sp.* lipase¹ was added to a corn-soybean meal based diet containing 8% tallow (Table 5.1) at 0, 50, 100, 200 and 400 units per gram of diet. The diets were formulated to meet the nutrient requirements of broiler chickens as established by NRC (1994). The liquid lipase source was diluted in distilled water and sprayed on the diet during mixing in a small Hobart mixer. Dietary treatments, including the control diet with no added lipase, were sprayed with 40 ml water per kg of diet. All diets were stored at 4 C until use to avoid auto-oxidation. Chromic oxide was used as an indigestible marker.

Newly hatched commercial broiler chicks (Hubbard x Peterson) were randomly housed in battery cages with ten chicks assigned to each replicate cage and six cages to each treatment. At 21 days of age the chickens were transferred to larger grower cages; three chicks were assigned to each cage and five replicates to each treatment. The chicks were exposed to constant light and provided feed and water on an *ad libitum* basis. Room temperature was gradually reduced from 34.5 to 22 C between 1 and 28 days of age and remained constant thereafter.

Pen feed consumption and body weight were recorded weekly until the termination of the experiment at 35 days of age. At 7, 14, 21 and 35 days of age, 2

¹ Finnfeeds International Inc., Marlborough, Wiltshire, SN8 1AA UK.

TABLE 5.1. Composition and nutrient content of basal diets

Ingredient	Content (g/kg)	
	Age fed (d)	
	0-21	21-35
Corn	478.01	574.75
Soybean meal (48%)	394.99	303.49
Tallow	80.00	80.00
Limestone	16.61	15.35
Dicalcium phosphate	16.25	14.36
Chromic oxide	5.00	5.00
Salt	4.65	3.45
DL Methionine	1.79	0.90
Choline chloride	1.00	1.00
Vitamin premix ¹	1.00	1.00
Mineral premix ²	0.70	0.70
Total	1000.00	1000.00
Nutrients (calculated)		
ME, kcal/kg	3109	3210
Crude protein, %	23.00	19.39
Crude fat, % (measured)	11.86	12.44
Ca, %	1.00	0.90
P, Avail., %	0.45	0.40
Na, %	0.20	0.16
Met, %	0.53	0.40
Met + Cys, %	0.90	0.72
Lys, %	1.29	1.28

¹ Provides per kilogram of diet: vitamin A, 8050 IU; vitamin D₃, 1820 ICU; vitamin E, 28 IU; menadione, 1.8 mg; thiamine, 1.8 mg; riboflavin, 7 mg; niacin, 58.8 mg; pyridoxine, 2.8 mg; vitamin B₁₂, 0.01 mg; pantothenic acid, 10.8 mg; folic acid, 0.77 mg; biotin, 98 mg; antioxidant, 87.5 mg.

² Provides per kilogram of diet: Fe, 56 mg; Zn, 77 mg; Mn, 94.5 mg; Cu, 7 mg; I, 0.91 mg; Se, 0.21 mg.

birds from each replicate were killed using intravenous sodium pentobarbitol injection to minimize peristaltic movement of the gastrointestinal tract and contents (Badawy, 1964). Duodenal and jejunal digesta from the two birds of each replicate were pooled, weighed, placed on dry ice for rapid freezing and then stored at -20 C for later analysis. The pancreata from these birds were collected and stored in a similar fashion. Samples of feces were collected twice per day on days 5-7, 12-14, 19-21 and 33-35, and stored at -20 C for future analyses.

Feed and feces samples were dried in a forced-air oven at 60 C for 72 hours, ground and then stored until required for analysis. The AOAC method (1980, Secs. 7.055-7.056) using Goldfish apparatus was used to extract fat from feed and feces samples. The chromic oxide content of feed and feces samples was measured according to the procedure of Fenton and Fenton (1979). Fatty acids were extracted and esterified (Metcalf and Wang, 1981) prior to measuring the fatty acid profile of individual samples using gas chromatography. Each sample was assayed twice. The instrument used was a Varian model 5890 series II with a flame ionization detector and capillary column² (30 m length and 0.25 mm internal diameter). The column oven temperature was 210 C. The injector and detector temperature was 250 C and the injector port was equipped with a splitter with a 50:1 split ratio. The flow rate of the He carrier gas was adjusted to 12 Psi pressure (1 ml/min), air at 500 ml/min, and hydrogen at 30 ml/min. The peak areas for each

² DB-23, J & W Scientific Specialties Inc., Brockville, Ontario K6V 5W1 Canada.

fatty acid were measured using a Hewlett-Packard 3396 series II integrator³ and expressed as percentages of the total fatty acids detected. Identification of the peaks was made on the basis of the retention times of standard methyl esters of individual fatty acids⁴ (appendices I-III). The amount of tri, di, monoglyceride and free fatty acid content of tallow and diets was tested by HPLC⁵ using low molecular weight gel permeation chromatography with a Waters Styragel HR 0.45, 7.8 x 300 mm column (appendices IV-VII). The mobile phase was 100% tetrahydrofuran at 1 ml/min. The samples were assessed by an Evaporative Light Scattering Detector⁵. The column and detector temperatures were 30 and 40 C, respectively.

The pooled duodenal and jejunal contents were freeze dried and 0.5 g of dried sample was mixed with 5 ml of ice cold distilled water and vortexed for 5 seconds. The vials were then centrifuged for 10 min at 2000 rpm and 4 C, and the supernatant collected in 1 ml vials for determining lipase activity. The rate of triglyceride hydrolysis was measured titrimetrically by the quantity of NaOH added per unit of time required to maintain a constant pH of 7 as described previously (Chapter 3).

Data were analyzed statistically by both regression analysis and analysis of variance using the Statistical Analytical Systems (SAS Institute, 1985) as a

³ Hewlett-Packard, Avondale, PA 19311-9990 USA.

⁴ Nu-Check-Prep. Inc., Elysian, MN 56028 USA.

⁵ M. Reaney, POS Pilot Plant Corp., 118 Veterinary Road, Saskatoon, SK., S7N 2R4 Canada.

completely randomized design. When treatment effects were significant, Duncan's multiple range test (Steel and Torrie, 1980) was used to compare means.

5.4 Results

The effect of dietary supplementation of *Pseudomonas sp.* lipase on body weight gain in chickens from 1 to 35 days of age is shown in Table 5.2. A negative effect of dietary lipase on body weight gain was seen at all ages. Regression analyses demonstrated that the negative effect of lipase addition was significant for all time periods except days 0-7 and 28-35. A quadratic relationship was shown for 7-14 and 1-21 days of age. Dietary lipase decreased feed intake for all time periods but the effect was only significant for 0-7, 14-21 and 1-21 days of age as indicated by ANOVA and/or regression analyses (Table 5.3).

Feed to gain ratio increased with increasing levels of lipase for 7-14, 21-28 and 21-35 days of age (Table 5.4). The response was quadratic for week 2. A similar response was noted for 1-21 days of age but failed to reach significance as judged by $P < 0.05$.

Actual pancreatic weights were unaffected by dietary lipase but a positive linear relationship was found between lipase supplementation and proportional pancreatic weight at 14 and 35 days of age (Table 5.5). Lipase activity in samples of pooled duodenal and jejunal contents was not affected by dietary treatment at 7 days of age (Table 5.6). Supplemental enzyme addition caused a quadratic

TABLE 5.2. The effect of dietary *Pseudomonas* sp. lipase on body weight gain in broiler chickens

Treatment ¹	Body weight gain (g)					
	0-7 d	7-14 d	14-21 d	21-28 d	28-35 d	1-21 d
0	105.2	239.2 ^a	383.0	500.6	574.3	727.3 ^a
50	104.0	199.0 ^b	355.6	502.2	566.6	658.6 ^b
100	107.6	203.8 ^b	378.7	473.2	589.3	690.1 ^b
200	102.4	188.2 ^b	348.0	473.5	524.6	638.6 ^c
400	101.7	193.7 ^b	354.3	392.5	538.7	649.7 ^c
SEM	3.24	6.92	10.48	31.26	18.96	12.10
Probability	0.722	0.0002	0.090	0.127	0.141	0.0001
Contrast	P value					
Regression						
Linear	0.3359	0.0010	0.0837	0.0124	0.0726	0.0006
Quadratic	0.9066	0.0012	0.2781	0.5935	0.5100	0.0059

¹ Supplemental lipase, units/g of diet.

^{a-c} Means in columns with different superscripts are significantly different (P<0.05).

TABLE 5.3. The effect of dietary *Pseudomonas* sp. lipase on feed intake in broiler chickens

Treatment [†]	Feed intake (g)					
	0-7 d	7-14 d	14-21 d	21-28 d	28-35 d	21-35 d
0	132.4	325.0	579.1 ^a	809.6	1021.2	1036.4 ^a
50	128.0	304.3	538.7 ^b	863.7	1012.6	971.1 ^b
100	133.2	313.1	565.5 ^{ab}	851.1	981.2	1011.8 ^{ab}
200	127.9	299.8	527.9 ^b	833.1	945.0	955.5 ^b
400	121.6	301.3	543.7 ^{ab}	757.9	955.5	966.7 ^b
SEM	3.61	7.90	11.97	47.64	37.54	18.25
Probability	0.201	0.170	0.036	0.559	0.536	0.020
Contrast	-----P value-----					
Regression						
Linear	0.0371	0.0828	0.0898	0.2160	0.1586	0.0255
Quadratic	0.6163	0.2132	0.0759	0.3232	0.3471	0.1078
						0.8833

[†] Supplemental lipase, units/g of diet.

^{a,b} Means in columns with different superscripts are significantly different (P<0.05).

TABLE 5.4. The effect of dietary *Pseudomonas* sp. lipase on feed to gain ratio in broiler chickens

Treatment ¹	Feed to gain ratio					
	0-7 d	7-14 d	14-21 d	21-28 d	28-35 d	21-35 d
0	1.261	1.368 ^b	1.519	1.634	1.779	1.425
50	1.232	1.531 ^a	1.519	1.721	1.788	1.475
100	1.242	1.539 ^a	1.502	1.818	1.672	1.467
200	1.253	1.607 ^a	1.518	1.760	1.804	1.497
400	1.202	1.556 ^a	1.534	1.969	1.774	1.488
SEM	0.036	0.051	0.041	0.083	0.051	0.019
Probability	0.801	0.034	0.988	0.098	0.403	0.111
Contrast	-----P value-----					
<u>Regression</u>						
Linear	0.3362	0.0449	0.7207	0.0124	0.7839	0.0575
Quadratic	0.7114	0.0168	0.7630	0.9488	0.7256	0.0907

¹ Supplemental lipase, units/g of diet.

^{a,b} Means in columns with different superscripts are significantly different (P<0.05).

TABLE 5.5. The effect of dietary *Pseudomonas sp.* lipase on actual and proportional pancreas weight in broiler chickens

Treatment ¹	Pancreas weight (g)				(Pancreas/body weight) * 1000			
	7 d	14 d	21 d	35 d	7 d	14 d	21 d	35 d
0	1.59	3.59	5.51	9.03	5.77	4.72	3.51	2.45
50	1.68	3.59	5.52	9.66	5.78	5.19	3.80	2.72
100	1.52	3.46	5.48	9.67	5.39	5.08	3.64	2.61
200	1.58	3.65	5.64	9.49	5.99	5.36	3.97	2.83
400	1.67	3.62	5.18	9.65	5.94	5.44	3.61	2.90
SEM	0.083	0.194	0.300	0.405	0.355	0.224	0.237	0.110
Probability	0.880	0.965	0.857	0.764	0.776	0.211	0.686	0.060
Contrast	-----				(P value)	-----		
<u>Regression</u>								
Linear	0.7308	0.7989	0.4350	0.5241	0.5348	0.0492	0.8668	0.0113
Quadratic	0.5762	0.9321	0.4957	0.5784	0.8911	0.3211	0.2293	0.3271

¹ Supplemental lipase, units/g of diet.

TABLE 5.6. The effect of dietary *Pseudomonas sp.* on the lipase activity of pooled duodenal and jejunal contents in broiler chickens

Treatment ¹	Lipase activity ²			
	7 d	14 d	21 d	35 d
0	364.4	580.5	563.5 ^a	667.1
50	383.8	662.5	551.9 ^a	561.7
100	393.3	741.0	418.8 ^b	638.5
200	407.6	700.2	513.4 ^{ab}	486.7
400	353.8	647.0	405.4 ^b	435.2
SEM	47.55	37.79	38.84	67.22
Probability	0.932	0.066	0.022	0.114
Contrast	-----	(P value)	-----	-----
<u>Regression</u>				
Linear	0.7988	0.5972	0.0131	0.0175
Quadratic	0.3917	0.0124	0.6807	0.6656

¹ Supplemental lipase, units/g of diet.

² Micro mol free fatty acids released/min/g dry matter of digesta.

^{a,b} Means in columns with different superscripts are significantly different (P<0.05).

increase in lipase activity at 14 days of age. At 21 and 35 days of age, the addition of lipase caused a linear decrease in lipase activity.

Dietary supplementation of *Pseudomonas sp.* lipase caused a linear reduction of fat digestibility at 19-21 and 33-35 days of age (Table 5.7). A similar trend was also seen at 0-7 days of age but the effect was not significant

The fatty acid composition of tallow, and the broiler starter and grower diets, are shown in Table 5.8. The major fatty acids in tallow were C16:0, C18:0 and C18:1; the diet profiles were similar but contained a higher proportion of 18:2. Minor fatty acids (less than 1% except for C18:3) are not included in this table.

The effects of 400 units per gram of dietary lipase on the digestibility of individual fatty acids are shown in Table 5.9. Lipase tended to decrease the digestibility of C14:0, C16:0 and C18:3 and increase the digestibility of C18:0, C18:1 and C18:2 at 5-7 days of age. At 19-21 and 33-35 days of age, lipase decreased the digestibility of C14:0, C16:1, C18:1 and C18:3 while increasing the digestibility of C16:0, C18:0 and C18:2.

Samples of the tallow and starter diets containing 0 and 400 units of lipase per gram of diet were analyzed for tri, di and monoglycerides as well as free fatty acids. The tallow and diet without enzyme contained predominately triglycerides (99%) but fat in the diet supplemented with lipase was primarily (83%) in the form of free fatty acids (Table 5.10). Smaller amounts of tri and diglycerides were also found in the enzyme supplemented diet.

TABLE 5.7. The effect of dietary *Pseudomonas sp.* lipase on the apparent fat digestibility in broiler chickens

Treatment ¹	Fat digestibility (%) ²		
	5-7 d	19-21 d	33-35 d
0	42.7	83.5 ^a	89.4 ^a
50	34.5	72.0 ^b	86.1 ^{bc}
100	31.3	71.8 ^b	87.8 ^{ab}
200	35.7	72.8 ^b	86.2 ^{bc}
400	36.8	65.6 ^b	84.8 ^c
SEM	4.00	2.88	0.92
Probability	0.375	0.004	0.032
Contrast	-----	(P value)	-----
<u>Regression</u>			
Linear	0.7086	0.0014	0.0057
Quadratic	0.1275	0.2758	0.4981

¹ Supplemental lipase, units/g of diet.

^{a-c} Means in columns with different superscripts are significantly different (P<0.05).

² Apparent fat digestibility calculated from the formula (AD=100 - (% marker in feed / % marker in feces) x (% nutrient in feces / % nutrient in feed) x 100)) as described by Saha and Gilbreath (1993).

TABLE 5.8. Fatty acid composition of tallow and basal diets fed to broiler chickens (%)

Fatty acids	Tallow	Diets	
		1-21 d	21-35 d
C14:0	3.41	2.65	2.42
C16:0	24.52	20.80	20.87
C16:1	4.42	3.48	3.41
C18:0	15.55	13.28	12.98
C18:1	38.06	36.68	37.38
C18:2	2.26	11.46	12.68
C18:3	0.38	0.82	0.77
U/S ¹	1.04	1.43	1.50

¹U/S, unsaturated to saturated fatty acids.

TABLE 5.9. The effect of dietary *Pseudomonas sp.* lipase on the digestibility of individual fatty acids in broilers (%)¹

Fatty acids	5-7 d		19-21 d		33-35 d	
	Control	400 U/g diet	Control	400 U/g diet	Control	400 U/g diet
C14:0	94.83±0.52	56.94±3.13	93.06±0.11	64.80±0.98	91.15±0.25	73.60±3.28
C16:0	82.73±0.13	62.42±0.96	71.16±0.08	76.04±0.28	68.53±1.18	76.22±1.21
C16:1	39.98±11.22	41.85±6.60	59.29±0.34	38.63±0.03	70.57±0.58	60.68±1.39
C18:0	60.86±3.38	69.62±0.89	58.13±0.43	74.59±0.68	57.83±1.80	72.14±1.23
C18:1	59.85±0.45	63.71±0.97	68.55±0.31	56.36±0.12	76.72±1.03	66.61±1.26
C18:2	57.97±0.49	68.12±0.43	43.39±0.64	72.77±0.46	67.42±1.24	71.74±0.73
C18:3	71.41±0.12	63.92±0.45	72.89±0.03	68.72±1.08	87.86±1.04	50.57±4.14

¹ Apparent digestibility of individual fatty acids calculated from the formula (AD=100 - (% marker in feed / % marker in feces) x (% nutrient in feces / % nutrient in feed) x 100)) as described by Saha and Gilbreath, 1993).

TABLE 5.10. The amount of tri, di and monoglycerides and free fatty acids in tallow and diets containing 8% tallow with or without dietary *Pseudomonas sp.* lipase¹ (%)

Samples	Tallow	Diet without lipase	Diet with lipase
Triglycerides	100	99.47	9.45
Diglycerides	0	0	7.67
Monoglycerides	0	0	0
Free fatty acids	0	0.53	82.88

¹ 400 units/g of diet as liquid form.

5.5 Discussion

Preliminary *in vitro* experiments in our laboratory demonstrated that *Pseudomonas sp.* lipase was capable of hydrolyzing the triglycerides found in tallow. This result is in agreement with the results of Kosngi *et al.* (1988) who found that *Pseudomonas sp.* lipase hydrolyzed tallow better than other lipase sources tested. The hydrolytic ability of this enzyme source is also shown by the finding that it was active prior to feeding. Although the complete absence of water will inactivate lipase activity, levels as low as 1% will permit enzyme activity (Zaks *et al.*, 1984, 1985). The diets used in this study contained approximately 10% moisture which was obviously sufficient to allow triglyceride hydrolysis during feed mixing or storage prior to feeding. The very high level of free fatty acids in the feed also show that the *Pseudomonas sp.* lipase is capable of hydrolyzing all three positions on the glycerol molecule. The lack of positional specificity for lipase from organisms from this genus has been reported previously (Jaerger *et al.*, 1994).

The confirmation that *Pseudomonas sp.* lipase is capable of hydrolyzing triglycerides to free fatty acids may explain the generally negative effect of dietary lipase on broiler productivity seen in this study. Previous research has clearly demonstrated that diets containing high levels of free fatty acids decrease bird performance (Sklan, 1979; Wiseman and Salvador, 1991; Thacker *et al.*, 1994; Vila and Garcia, 1996). Sklan (1979) showed that the overall absorption of total

fatty acids was highest in triglyceride-fed chicks and lowest in chicks receiving free fatty acids. He emphasized the need for monoglycerides to facilitate the efficient solubilization and absorption of free fatty acids. The reduction in apparent fat digestibility shown for lipase supplementation in this study support this concept.

The fatty acid profile of tallow in the present study was similar to previously reported values (Carlier *et al.*, 1991; Christie *et al.*, 1991; Gunstone *et al.*, 1994). The main fatty acids of tallow are palmitic (C16:0, mostly in position 1), stearic (C18:0, mostly in position 1 and 3) and oleic acid (C18:1, mostly in position 2). The results of this study show that a high level of lipase has variable effects on specific fatty acids. The generally increased digestibility of long chain saturated fatty acids (C16:0, C18:0) suggest that endogenous lipase may be deficient or less effective than the *Pseudomonas sp.* lipase in broiler chickens even at older ages. An alternate suggestion in relation to the effect of lipase digestibility of C16:0 and C18:0 would be the formation of soaps in the gut. Saturated fatty acids in comparison to unsaturated fatty acids, preferentially bind with cations such as Ca^{++} and form mineral soaps (Atteh and Leeson, 1984). Soaps may not have been extracted by di-ethyl ether (as used in this experiment) and as a consequence could have affected the digestibility results. The decrease in the digestibility of the predominate dietary fatty acid, oleic acid (18:1), may not be unexpected as most of this fatty acid is found in the 2-position in tallow and

therefore has the advantage of being a monoglyceride in normal pancreatic digestion of triglycerides in the chicken. Previous research has shown that monoglycerides are retained more efficiently than free fatty acids (Sklan, 1979). As a result of the non-specific nature of the *Pseudomonas sp.* lipase, oleic acid would be a free fatty acid and therefore have a poorer digestibility. The effects of lipase on the apparent digestibility of individual fatty acids does not correspond well to the fat digestibility results. This may be due to the properties of the solvent used for fat and fatty acid extraction (di-ethyl ether). Di-ethyl ether is a non-polar solvent (Waczkowski *et al.*, 1989) and extracts both polar (phospholipids and glycolipids) and non-polar (tri, di and monoglycerides, fatty acids, sterols and hydrocarbons) compounds (Hoseney *et al.*, 1969). As an example, the lipid extracted from lentil flour by petroleum ether consists of 78.3% of neutral lipids, 14.9% phospholipids and 6.8% glycolipids (Waczkowski *et al.*, 1989). Therefore, lipids other than triglycerides may have affected the apparent fat digestibility results in the present study.

The effect of the dietary enzyme supplementation on lipase activity in the small intestine demonstrates that the dietary source was in fact contributing to the supply of this enzyme class in the intestine of the young bird (at least at two weeks of age). However, at older ages, dietary lipase caused a decrease in digestive tract lipase activity. Changes in pancreatic flow, bile flow and/or bile precipitation may explain these results as free fatty acids affect these characteristics.

In theory, feeding free fatty acids decreases the pH of intestinal contents which could have an important impact on digestion and utilization of fat. Reducing the pH in the small intestine could reduce the activity of lipase and /or result in bile salt precipitation (DiMagno, 1982). Therefore, it can be speculated that even a minimal reduction in the secretion of bile acids, if coupled with intraluminal acidic precipitation of bile salts, could lead to fat malabsorption.

Dietary lipase increased proportional pancreatic weight, albeit not consistently. This does not support the concept of free fatty acids causing a reduction in pancreatic secretion and suggests that there may be specific inhibition of lipase in the small intestine as has been well documented for enzyme inhibitors found in raw soybeans (Khalifa *et al.*, 1994). It is of interest to note that feeding exogenous amylases and proteases have been shown to cause pancreatic enlargement (Mahagna *et al.*, 1995). These results suggest caution when using dietary enzymes which mimic endogenous enzymes. The inherent control mechanisms found in the animal may produce less desirable effects than anticipated from the exogenous source. If this scenario is valid, the dose of exogenous enzyme would be important. Because of the lack of research on the use of dietary lipase, the authors had no valid way of establishing enzyme levels in this research and may have exceeded the required or desirable inclusion level.

Regardless of whether the free fatty acids caused the negative production effect, their presence in feed would not be desirable because of the increased

potential for oxidation. The diets in this research were kept in a cold room at 4 C to reduce this possibility but this is unacceptable in commercial practice.

5.6 Conclusions

In summary, dietary supplementation with a liquid form of *Pseudomonas* *sp.* lipase caused a decrease in the performance of young broiler chickens. It is hypothesized that the negative effect of this lipase is due to the hydrolysis of triglycerides to free fatty acids (even in the feed) which in turn reduces fat digestibility. The results suggest that for a lipase to be suitable for use as a dietary supplement, it should not be active in feed and should not hydrolyze triglycerides completely to free fatty acids. Enzyme dosage and form (liquid or dry) may also have an impact on the results of using dietary lipase in young chickens.

6.0 THE EFFECT OF DIETARY SUPPLEMENTATION OF *PSEUDOMONAS SP.* LIPASE ON FAT DIGESTIBILITY IN TURKEY POULTS

6.1 Abstract

An *in vivo* experiment was conducted to study the use of dietary *Pseudomonas sp.* lipase to improve fat digestibility by young turkeys from 0-21 days of age. In a completely randomized experiment, 112 day-old Large White Hybrid male turkey poults were assigned to battery cages with 4 per cage and 8 replicates per treatment. A commercial liquid form of *Pseudomonas sp.* lipase was added at 0, 20, 40 and 60 units per gram of diet to a corn-soybean meal based diet containing 8% pure beef tallow. Feed and water were provided *ad libitum*. Acid insoluble ash was used as indigestible marker for fat and fatty acid digestibilities. Samples of feces were collected at 5-7, 12-14 and 19-21 days of age and dried for digestibility status of fat and fatty acids. Dietary *Pseudomonas sp.* lipase decreased body weight gain from 0-7, 7-14 and 0-21 days of age and feed intake from 7-14 and 0-21 days of age. Lipase addition increased feed to gain ratio from 0-7 days of age but had no effect for other time periods. Enzyme use did not affect overall fat digestibility. However, the dietary lipase increased the digestibility of C18:0 and decreased the digestibility of C14:0 and C18:1. It was concluded that dietary supplementation of *Pseudomonas sp.* lipase did not

improve the overall fat digestibility and performance of young turkey poults but has the potential to improve the digestibility of long chain saturated fatty acids.

6.2 Introduction

Fats are often a cost effective energy source in poultry diets and provide other beneficial effects in feed manufacture and poultry production. A factor limiting the use of fats in poultry diets is the relatively poor ability of young birds to use fats and in particular, to use fats containing a high proportion of saturated fatty acids (Sell *et al.*, 1986; Lesson and Atteh, 1995). The reason for the poor fat digestibility in young birds has not been established but the immaturity of the digestive system has received most attention (Chapter 5).

A lipase deficiency has been suggested as one of the factors that might cause poor fat utilization in young birds. This suggestion is based on the finding that the lipase concentration in the small intestine is low in young birds and increases markedly with age (Krogdahl and Sell, 1989; Pubols, 1991). Dietary supplementation with pancreatic lipase offers the potential to increase fat utilization in the case of pancreatic lipase deficiency. Dietary pancreatic lipase has been used in young chickens (Polin *et al.*, 1980) and in humans (DiMagno *et al.*, 1973) but failed to eliminate fat malabsorption in these species. Polin *et al.* (1980) reported that dietary supplementation of crude porcine lipase (steapsin) in a corn-soybean meal based diet containing 4% tallow could not eliminate

steatorrhoea in young chickens. Destruction of the pancreatic lipase in the stomach and/or small intestine, and variability in the potency of commercial lipase supplements (DiMagno *et al.*, 1977; Raimondo and DiMagno, 1994) are the possible reasons for the failure of dietary lipase to overcome steatorrhoea.

In vitro studies have tested the activity of several lipase sources under conditions which approximate the proventriculus and small intestine of young birds (Chapters 3 and 4). It was shown that the bacterial *Pseudomonas sp.* lipase was relatively stable to the conditions of the digestive tract and showed promise as an additive to poultry diets. However, *in vivo* research with broiler chickens demonstrated that the *Pseudomonas sp.* lipase caused a negative effect on broiler performance and did not improve fat digestibility (Chapter 5). Because of the minimal research on the use of dietary lipase, the dosage of enzyme was chosen arbitrarily and may have been excessive. In addition, it is possible that other avian species like the turkey may be affected differently by the use of dietary lipase to improve fat digestibility. Therefore, the present study was designed to establish the effect of lower levels of dietary lipase (*Pseudomonas sp.*) than those used in chicken diets (Chapter 5) on the performance and fat digestibility of turkey poults.

6.3 Materials and Methods

Large White Hybrid turkey poults were used to test the effect of dietary *Pseudomonas sp.* lipase¹ on fat digestibility and bird performance. The liquid lipase was added to a corn-soybean meal based diet containing 8% pure beef tallow (Table 6.1) at 0, 20, 40 and 60 units per gram of diet. Lipase units were established according to the procedure described in Chapter 3. The basal diet was formulated according to NRC (1994) to meet or exceed the requirements of turkey poults. The liquid lipase source was diluted in distilled water and sprayed on the diet during mixing in a small Hobart mixer. Dietary treatments, including the control diet with no added lipase, received 40 ml of water per kg of diet. All diets were stored at 4 C until use to avoid auto-oxidation. The diets contained acid insoluble ash as an indigestible marker.

Day-old poults were randomly assigned to battery cages with four per cage and eight replications per treatment. Feed and water were provided *ad libitum*. Room temperature was initially 34.5 C and was gradually reduced to 24.5 C by 21 days of age. The birds were given constant light (24L:0D). Pen feed consumption and body weights were measured weekly and feces samples were collected two times per day at 5-7, 12-14 and 19-21 days of age. The feces were stored at -20 C. Feed and feces samples were dried in a forced-air oven at 60 C for 72 hours, ground and then stored for later analysis. The AOAC method (1980, secs. 7.055-

¹ Finnfeeds International Inc., Marlborough, Wiltshire, SN8 1AA U.K.

TABLE 6.1. Composition and nutrient content of basal diets

Ingredient	Content (g/kg)
	Age fed (0-21 d)
Soybean meal (48%)	510.80
Corn	343.10
Tallow	80.00
Dicalcium phosphate	35.30
Limestone	12.60
Acid insoluble ash	5.00
Vitamin mineral premix ¹	5.00
Salt	3.90
DL Methionine	2.40
L-Lysine HCL	1.80
Ethoxyquin	0.20
Total	1000.10
Nutrients (calculated)	
ME, kcal/kg	3033
Crude protein, %	28.00
Crude fat, % (measured)	10.88
Ca, %	1.40
P, Avail., %	0.80
Na, %	0.17
Met, %	0.63
Met + Cys, %	1.00
Lys, %	1.80

¹ Provides per kilogram of diet: vitamin A, 11000 IU; vitamin D₃, 2200 ICU; vitamin E, 30 IU; menadione, 2 mg; thiamine, 1.5 mg; riboflavin, 6 mg; niacin, 60 mg; pyridoxine, 4 mg; vitamin B₁₂, 0.02 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; antioxidant, 0.62 mg; Fe, 80 mg; Zn, 80 mg; Mn, 80 mg; Cu, 10 mg; I, 0.8 mg; Se, 0.3 mg.

7.056) using Goldfish apparatus was used to extract fat from feed and feces samples. The acid insoluble ash content of feed and feces samples was measured according to the procedure of McCarthy *et al.* (1974). Fatty acids were extracted, esterified (Metcalf and Wang, 1981) and then measured by gas chromatography. Each sample was assayed twice using a Varian model 5890 series II instrument with flame ionization detector and capillary column² (30 m length and 0.25 mm internal diameter). The column oven temperature was 210 C. The injector and detector temperature was 250 C and the injector port was equipped with a splitter with a 50:1 split ratio. The flow rate of the He carrier gas was adjusted to 12 Psi pressure (1 ml/min), air at 500 ml/min, and hydrogen at 30 ml/min. The peak areas for each fatty acid were measured using a Hewlett-Packard 3396 series II integrator³ and were expressed as percentages of the total fatty acids detected. Identification of the peaks was made on the basis of the retention times of standard methyl esters of individual fatty acids⁴. The amounts of tri, di, monoglyceride and free fatty acids of tallow and diets were measured by HPLC⁵ using low molecular weight gel permeation chromatography with a Waters Styragel HR 0.45, 7.8 x 300 mm column. The mobile phase was 100% tetrahydrofuran at 1 ml/min. The samples were detected by a Evaporative Light Scattering Detector⁵. The column and detector temperatures were 30 and 40 C, respectively.

² DB-23, j & W Scientific Specialties Inc., Brockville, Ontario K6V 5W1 Canada.

³ Hewlett-Packard, Avondale, PA 19311-9990 USA.

⁴ Nu-Check-Prep. Inc., Elysian, MN 56028 USA.

⁵ M. Reaney, POS Pilot Plant Corp., 118 Veterinary Road, Saskatoon, SK., S7N 2R4 Canada

Data were analyzed by both regression analysis and analysis of variance according to the general linear model (GLM) procedure of SAS[®] (SAS Institute, 1985) as a completely randomized design. When treatment effects were significant, Duncan's multiple range test (Steel and Torrie, 1980) was used to compare means. Statements of statistical difference are based on $P < 0.05$.

6.4 Results

Dietary *Pseudomonas* sp. lipase decreased the body weight gain of turkey poults in a linear fashion from 0 to 7, 7 to 14 and 0 to 21 days of age (Table 6.2). Feed intake was not affected by dietary lipase during week 1 and week 3 but was reduced linearly during week 2 and for the overall experimental period (Table 6.3). Feed to gain ratio increased in response to lipase level from 0 to 7 days of age but was not affected by treatment at other time periods (Table 6.4). Dietary lipase did not affect fat digestibility at any of the ages tested (Table 6.5).

The fatty acid composition of the tallow used in this research as well as the experimental diet are shown in Table 6.6. The major fatty acids in tallow were C16:0, C18:0 and C18:1. These fatty acids were similarly dominant in the diet, which also contained 9.96% C18:2. With the exception of C18:3, fatty acids which were less than 1% of the total were not included on the table.

The effect of dietary lipase (60 U/g diet) on the apparent digestibility of individual fatty acids is shown in Table 6.7. Lipase addition reduced the

TABLE 6.2. The effect of dietary *Pseudomonas sp.* lipase on body weight gain in turkey poult

Treatment ¹	Body weight gain (g)			
	0-7 d	7-14 d	14-21 d	1-21 d
0	111.9	234.1 ^a	291.5	637.5 ^a
20	102.4	212.9 ^b	272.2	587.4 ^b
40	106.7	212.0 ^b	285.1	603.8 ^{ab}
60	96.7	202.8 ^b	275.6	575.1 ^b
SEM	3.96	6.82	5.53	13.34
Probability	0.068	0.022	0.062	0.016
Contrast	(P value)			
<u>Regression</u>				
Linear	0.0272	0.0043	0.1568	0.0078
Quadratic	0.9553	0.3847	0.3641	0.4275

¹ Supplemental lipase, units/g of diet.

^{a,b} Means in columns with different superscripts are significantly different (P<0.05).

TABLE 6.3. The effect of dietary supplementation of *Pseudomonas sp.* lipase on feed intake in turkey poult

Treatment ¹	Feed intake (g/poult)			
	0-7 d	7-14 d	14-21 d	1-21 d
0 (control)	134.9	296.4 ^a	418.3	849.6
20	125.0	282.1 ^{ab}	396.5	803.7
40	132.4	281.8 ^{ab}	400.8	815.0
60	125.2	265.3 ^b	390.3	780.7
SEM	3.99	7.56	9.62	19.10
Probability	0.213	0.056	0.219	0.103
Contrast	(P value)			
<u>Regression</u>				
Linear	0.2301	0.0098	0.0735	0.0298
Quadratic	0.7419	0.8833	0.5622	0.7617

¹ Supplemental lipase, units/g of diet.

^{a,b} Means in columns with different superscripts are significantly different (P<0.05).

TABLE 6.4. The effect of dietary *Pseudomonas sp.* lipase on feed to gain ratio in turkey poult

Treatment ¹	Feed to gain ratio			
	0-7 d	7-14 d	14-21 d	1-21 d
0	1.207	1.268	1.435	1.333
20	1.226	1.324	1.456	1.367
40	1.243	1.330	1.407	1.351
60	1.302	1.318	1.417	1.359
SEM	0.025	0.024	0.023	0.014
Probability	0.072	0.249	0.462	0.325
Contrast	(P value)			
<u>Regression</u>				
Linear	0.0133	0.1501	0.3275	0.3109
Quadratic	0.4321	0.1620	0.8114	0.3356

¹ Supplemental lipase, units/g of diet.

TABLE 6.5. The effect of dietary *Pseudomonas* sp. lipase on the apparent fat digestibility in turkey poults

Treatment ¹	Fat digestibility (%) ²		
	5-7 d	12-14 d	19-21 d
0	77.9	53.0	78.3
20	78.4	52.3	80.7
40	76.5	47.7	80.2
60	79.5	57.8	80.1
SEM	1.57	4.11	1.48
Probability	0.615	0.419	0.680
Contrast	-----	(P value)	-----
<u>Regression</u>			
Linear	0.6874	0.6111	0.4819
Quadratic	0.4453	0.2096	0.3930

¹ Supplemental lipase, units/g of diet.

² Apparent fat digestibility measured from the formula (AD= 100 - (% marker in feed / % marker in feces) x (% nutrient in feces / % nutrient in feed) x 100)) as described by Saha and Gilbreath, 1993).

TABLE 6.6. Fatty acid composition of tallow and basal diet fed to turkey poult
(%)

Fatty acids	Tallow	Diet
C14:0	3.41	2.87
C16:0	24.52	21.80
C16:1	4.42	3.79
C18:0	15.55	13.46
C18:1	38.06	36.94
C18:2	2.26	9.96
C18:3	0.38	0.86
U/S ¹	1.04	1.35

¹U/S, unsaturated to saturated fatty acids.

TABLE 6.7. The effect of dietary *Pseudomonas sp.* lipase on digestibility of individual fatty acids in turkey poult chicks (%)¹

Fatty acids	5-7 d		12-14 d		19-21 d	
	Control	60 U/g diet	Control	60 U/g diet	Control	60 U/g diet
C14:0	90.53±0.05	54.41±1.30	88.07±0.60	62.01±2.24	92.86±0.01	31.79±2.53
C16:0	53.06±0.87	66.66±0.74	62.19±0.18	59.70±0.15	57.96±0.01	62.26±0.64
C16:1	52.92±1.21	65.56±0.50	43.66±2.01	39.63±10.51	65.50±5.80	50.02±8.02
C18:0	22.99±0.08	64.14±0.65	35.24±2.10	53.98±1.42	14.39±0.87	67.97±0.40
C18:1	74.97±0.42	53.98±1.12	49.75±0.70	55.71±1.62	75.62±0.22	57.33±0.56
C18:2	65.40±0.58	54.02±2.68	39.21±0.40	53.51±3.70	55.05±1.46	54.23±0.72
C18:3	82.09±0.56	36.72±6.45	65.14±0.87	36.25±15.30	75.81±2.32	53.86±0.14

¹ Apparent digestibility of individual fatty acids measured from the formula (AD= 100 - (% marker in feed / % marker in feces) x (% nutrient in feces / % nutrient in feed) x 100)) as described by Saha and Gilbreath, 1993).

digestibility of C14:0 and C18:1 and improved the digestibility of C18:0. The effect of lipase on digestibility of C16:0, C16:1 and C18:2 was minor and changed slightly with age.

6.5 Discussion

The use of *Pseudomonas sp.* lipase in this study reduced the growth rate of young turkey poult and failed to improve overall fat digestion. Despite the use of lower levels of enzyme, these results are similar to those previously shown for broiler chickens (Chapter 5). The ability of the *Pseudomonas sp.* lipase to hydrolyze triglycerides, and in particular those found in tallow, has been demonstrated previously (Kosngi *et al.*, 1988) and confirmed in the study with broiler chickens. The *Pseudomonas sp.* is also known to not be site specific and therefore can hydrolyze bonds in all three positions of the glycerol molecule. The result is the production of free fatty acids and glycerol in the absence of the monoglycerides that would be produced by the action of pancreatic lipase (Erlanson-Albertson, 1992). In the broiler study, a high level of *Pseudomonas sp.* lipase (400 U/g diet) was shown to have hydrolyzed triglycerides during diet mixing and/or storage, and prior to consumption. It is possible that the lower levels of lipase used in the present research produced the same effect.

It is well established that absorption of fatty acids is highest when fed in a triglyceride rather than free fatty acid form (Sklan, 1979; Wiseman and Salvador,

1991; Vila and Garcia, 1996). Sklan (1979) compared the overall absorption of total fatty acids in free and in triglyceride forms in chickens. He found that the absorption of fatty acids was highest in triglyceride-fed chicks and lowest in chicks receiving free fatty acids. Adequate amounts of monoglycerides for efficient solubilization and absorption of free fatty acids was suggested as the reason for this effect.

Dietary lipase seems to have a distinct effect on the digestibility of different fatty acids. The decrease in the digestibility of C18:1 in this study is similar to the results of feeding *Pseudomonas sp.* lipase to broiler chickens (Chapter 5). The majority of this fatty acid is found in the 2-position of tallow (Christie *et al.*, 1991) and it is possible that hydrolysis of this bond by the non-specific *Pseudomonas sp.* lipase is responsible for the reduced digestibility. It has been shown that in chickens the 2-monoglycerides promote the solubility and absorption of fatty acids (Garrett and Young, 1975). Sklan (1979) also showed that insufficiency of 2-monoglyceride in the intestinal lumen of chickens is responsible for an incomplete micellar solubilization of free fatty acids. The increase in the apparent digestibility of C18:0 also is in agreement with the broiler study. These data suggest that young poult and chicks are deficient in pancreatic lipase and therefore the addition of the exogenous lipase improved the utilization of C18:0. In contrast to the broiler study, the digestibility of C16:0 was not consistently improved by the addition of lipase.

Fat retention was lower from 12 to 14 than either 5 to 7 or 19 to 21 days of age. This trend was not seen in the broiler study (Chapter 5). It is possible that the ability of turkeys to digest fat decreases after hatch and then increases as the birds get older. The fact that intestinal lipase activity decreases between 4 and 10 days of age (Escribano *et al.*, 1988; Krogdahl and Sell, 1989; Sell *et al.*, 1991) supports this idea. One could also hypothesize that reduced fat digestibility from 12 to 14 days of age may be a secondary effect of infectious processes in the intestine. However, no clinical symptoms of disease such as diarrhea were observed.

6.6 Conclusions

In conclusion, dietary *Pseudomonas sp.* lipase added in the liquid form did not improve overall fat digestibility or performance of turkey poults between 0 and 21 days of age but did improve the digestibility of C18:0. Further research is required to clarify the importance of enzyme form (liquid or dry), the site of enzyme action (feed or digestive tract) and the site specificity of lipase sources (non-specific vs position 1 and 3 specificity) on the potential to use dietary lipase to improve fat digestibility in the young poult.

7.0 GENERAL SUMMARY AND CONCLUSIONS

The hypotheses of this thesis were that pancreatic lipase is deficient in young birds and results in low fat digestibility, and that a dietary source of lipase is capable of correcting this situation. An assumption of the initial research (Chapters 3 and 4) was that the lipase would need to be active in the bird's digestive tract. Therefore, lipase sources were evaluated for stability under conditions that mimic the glandular stomach and small intestine, and did not require cofactors such as colipase for activity. The results of this research demonstrate clear differences among lipase sources and may explain the inability of pancreatic lipase to dramatically improve fat digestion in previous avian research (Polin *et al.*, 1980). *Pseudomonas sp.* lipase was selected for *in vivo* study because of its relative superiority to remain active under the conditions of the digestive tract.

In vivo studies with both broiler chickens and turkeys failed to produce an improvement in overall fat utilization and indeed caused a decrease in bird productivity. However, it is premature to reject the thesis hypotheses. The ability of the *Pseudomonas sp.* lipase to be active in the diet prior to feeding was unexpected and is not well suited for a dietary lipase supplement. The increased potential for auto-oxidation of free fatty acids in comparison to triglycerides would make this finding unacceptable under commercial conditions. In addition, the non-specific nature of the *Pseudomonas sp.* lipase in regard to hydrolyzing bonds at all

three positions of the glycerol molecule and the resulting total hydrolysis of triglycerides would reduce fat utilization rather than improve it. This is related to the beneficial effects of monoglycerides in micelle formation and utilization of free fatty acids. Free fatty acids are not utilized as well as monoglycerides and the finding that the apparent digestibility of C18:1, which is primarily found in the 2 position of tallow triglycerides, was particularly reduced, support this concept. It is of interest that the apparent digestibility of C16:0 was improved by enzyme addition in the broiler experiment and that of C18:0 was improved in both the broiler and turkey trials. These findings suggest that lipase may be limiting in the utilization of long chain saturated fatty acids.

Dietary enzymes are used very successfully to overcome the antinutritional effects of fiber components found in cereal grains and to target specific compounds such as phytate. These enzymes are either not found in the digestive tract of the animal or are relatively ineffective (eg. phytase). Therefore, they are unlikely to influence the complex nature of the digestion of other dietary nutrients such as proteins, lipids and starch. On the other hand, dietary enzymes which mimic a similar activity in the animal's digestive tract may influence endogenous secretions via the effects of their hydrolytic products. The reduction of intestinal lipase activity and the increase in proportional pancreas weight during the later portion of the broiler experiment described in this thesis support this concept. It is

interesting that the use of exogenous protease and amylase have also been shown to reduce pancreatic secretion in chickens (Mahagna *et al.*, 1995).

An interesting question that arises from this thesis is “Where is the optimum place for lipase activity and triglyceride digestion?”. Since at least some lipases are active under minimal moisture conditions, hydrolysis could occur in the feed prior to feeding. As already discussed, this is not a desirable situation and probably should be avoided. Activity in the crop or proventriculus/gizzard is also possible; lingual and acidic lipases are found in other species (Borgstrom, 1986). It is interesting to note that these types of enzymes are particularly active on fats containing short to medium chain length fatty acids. One can speculate that this type of fat is selectively hydrolyzed because of the reduced requirement for micelle formation during absorption of these types of fatty acids. If this is an acceptable site for hydrolysis, the *Aspergillus niger* lipase tested in this study might be suitable as it showed higher activity at lower pH. It was originally assumed that the small intestine would be the optimum site of lipase activity because it is the site of absorption and bile secretions required to facilitate the process of digestion and absorption are also in this location. Although this study did not specifically address this possibility, it would seem that the original assumption is still correct.

Where the lipase is active may also be influenced by form. A liquid form was used in this study and this may have enhanced the lipase activity in the feed. Research is required to study the impact of lipase form on the location of its

activity. An enteric-coated microsphere formulation may be desirable to prevent activity until the small intestine and also to protect the lipase from the acidic conditions of the proventriculus and gizzard.

In addition to having a lipase that is not active until the small intestine, it seems that the production of monoglycerides is required for optimum digestion and absorption. Therefore lipases without hydrolysis site specificity are likely unacceptable as a dietary supplement.

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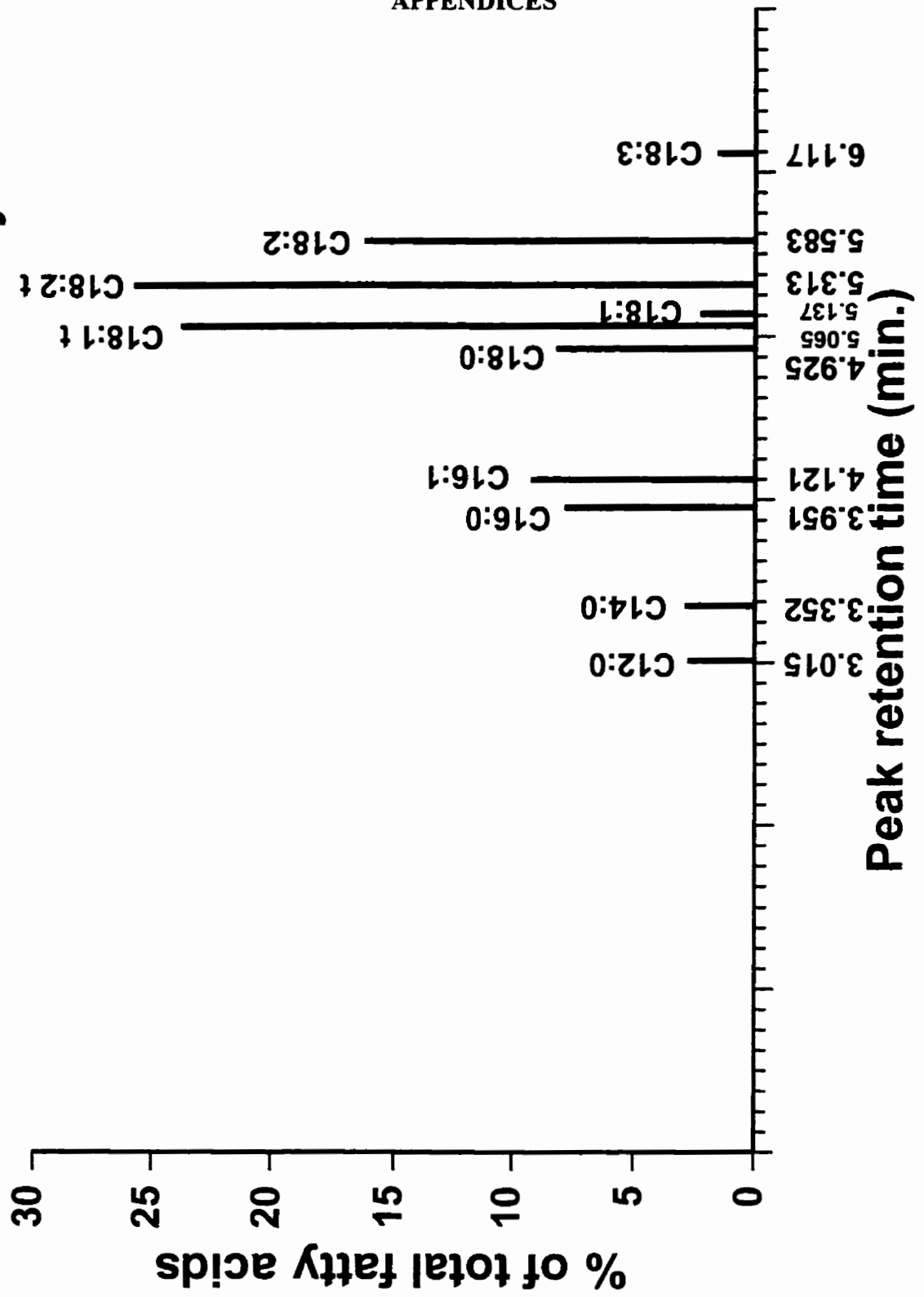
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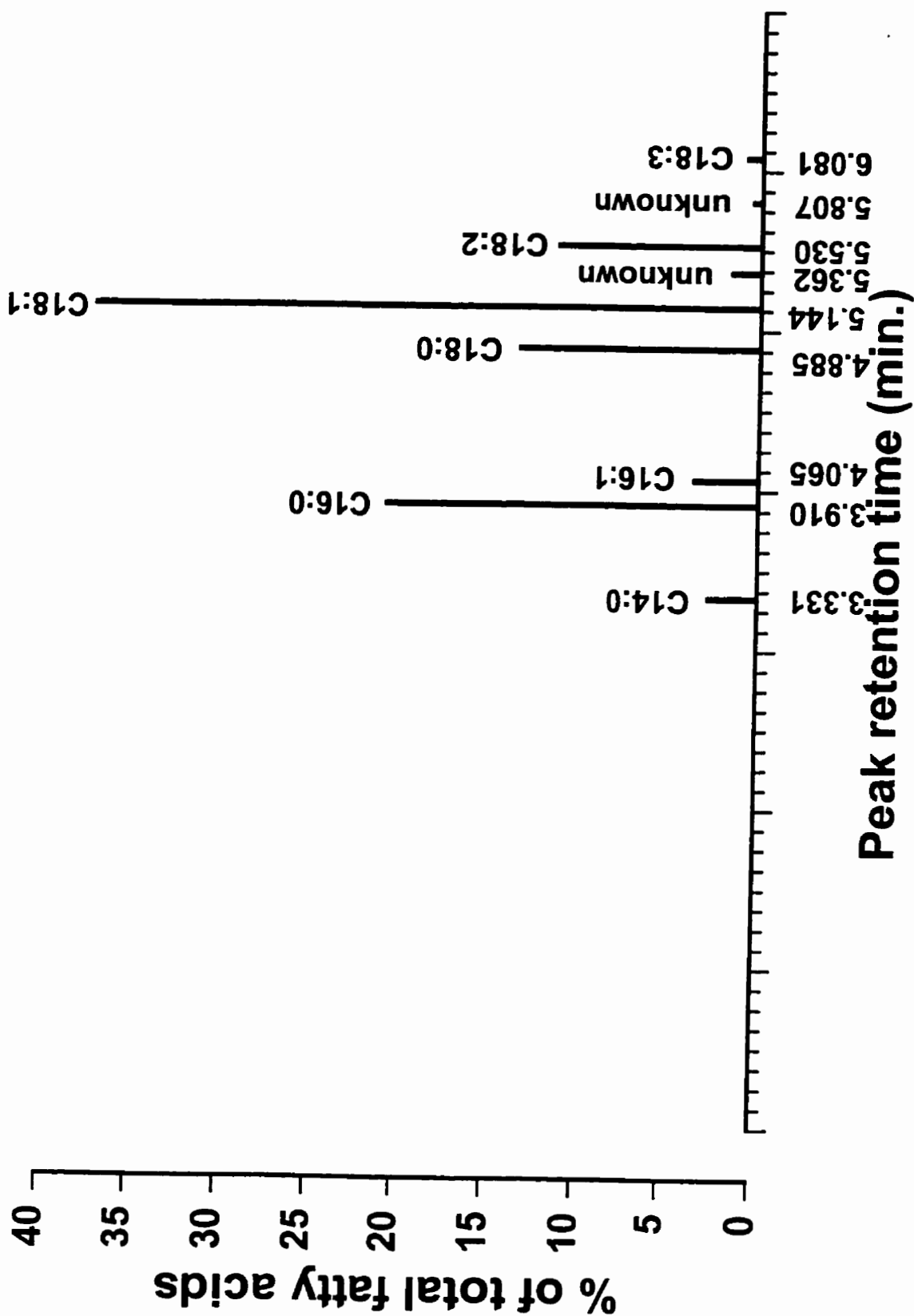
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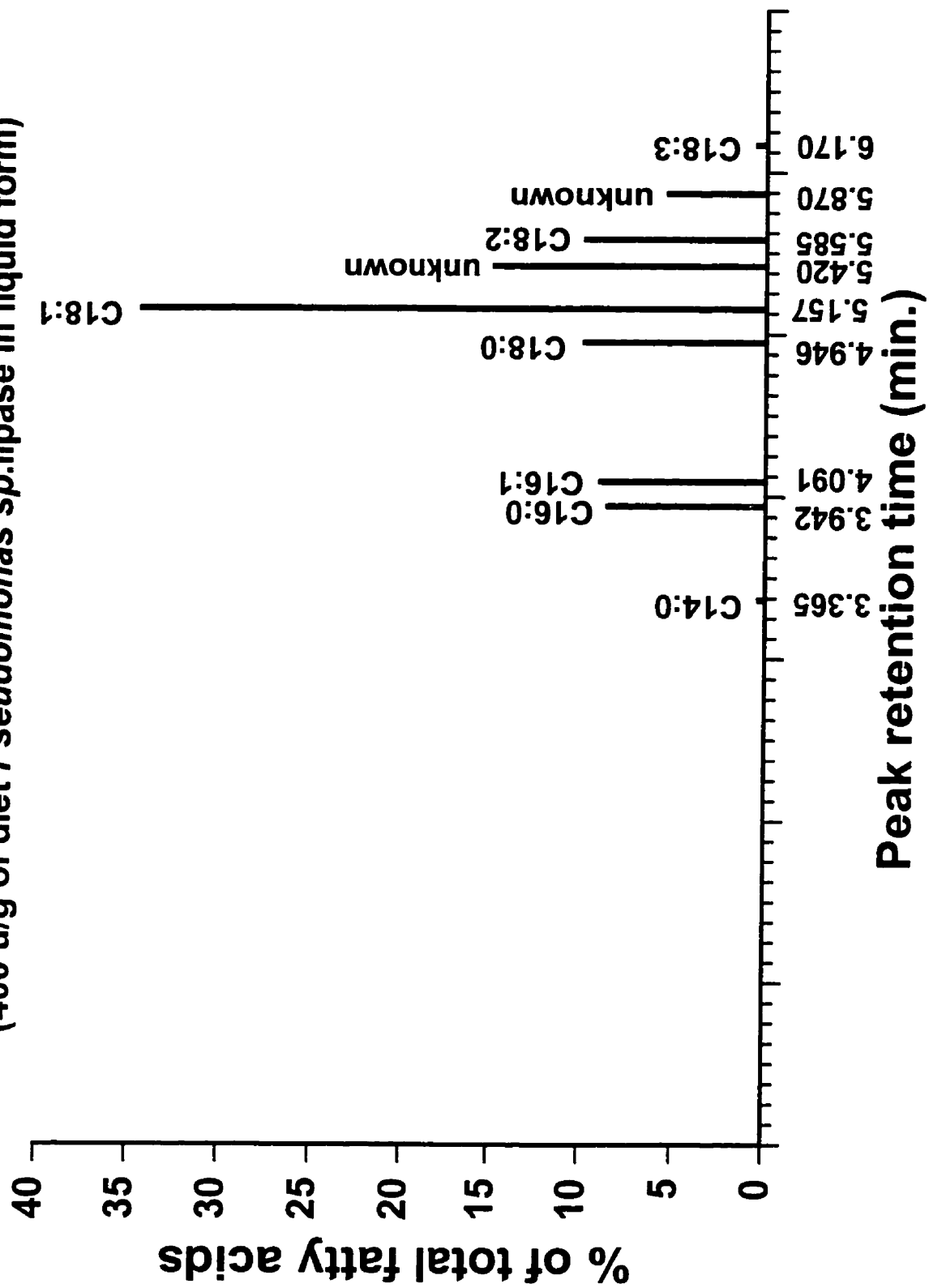
I. Peak retention times of standard fatty acids



II. Peak retention times of dietary fatty acids
(no added *Pseudomonas* sp. lipase)



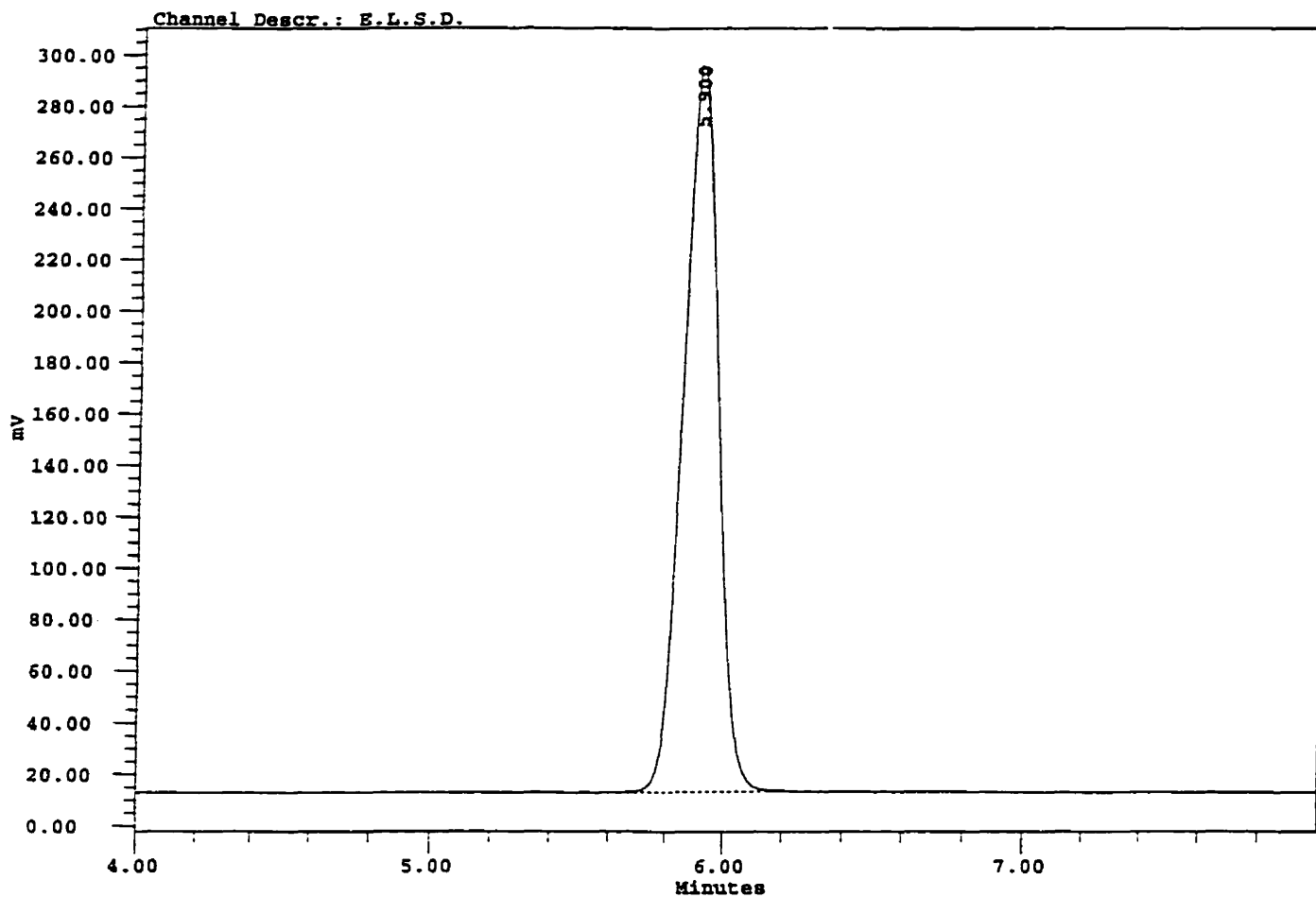
III. Peak retention times of feces fatty acids (400 u/g of diet *Pseudomonas* sp.lipase in liquid form)



ALLIANCE SAMPLE INFORMATION

Sample Name: #4 Tallow
 Vial: 52 Injection: 1
 Injection Volume: 5.00 μ L
 Date Acquired: 04/17/97 04:16:46 PM
 Project Name: Martin_Reaney

SampleInfo: Martin
 Run Time: 8.0 min
 Date Processed 04/17/97 04:25:06 PM
 Processing Method: GPC
 Acq Meth Set: GPC_Styrigel



Peak Results

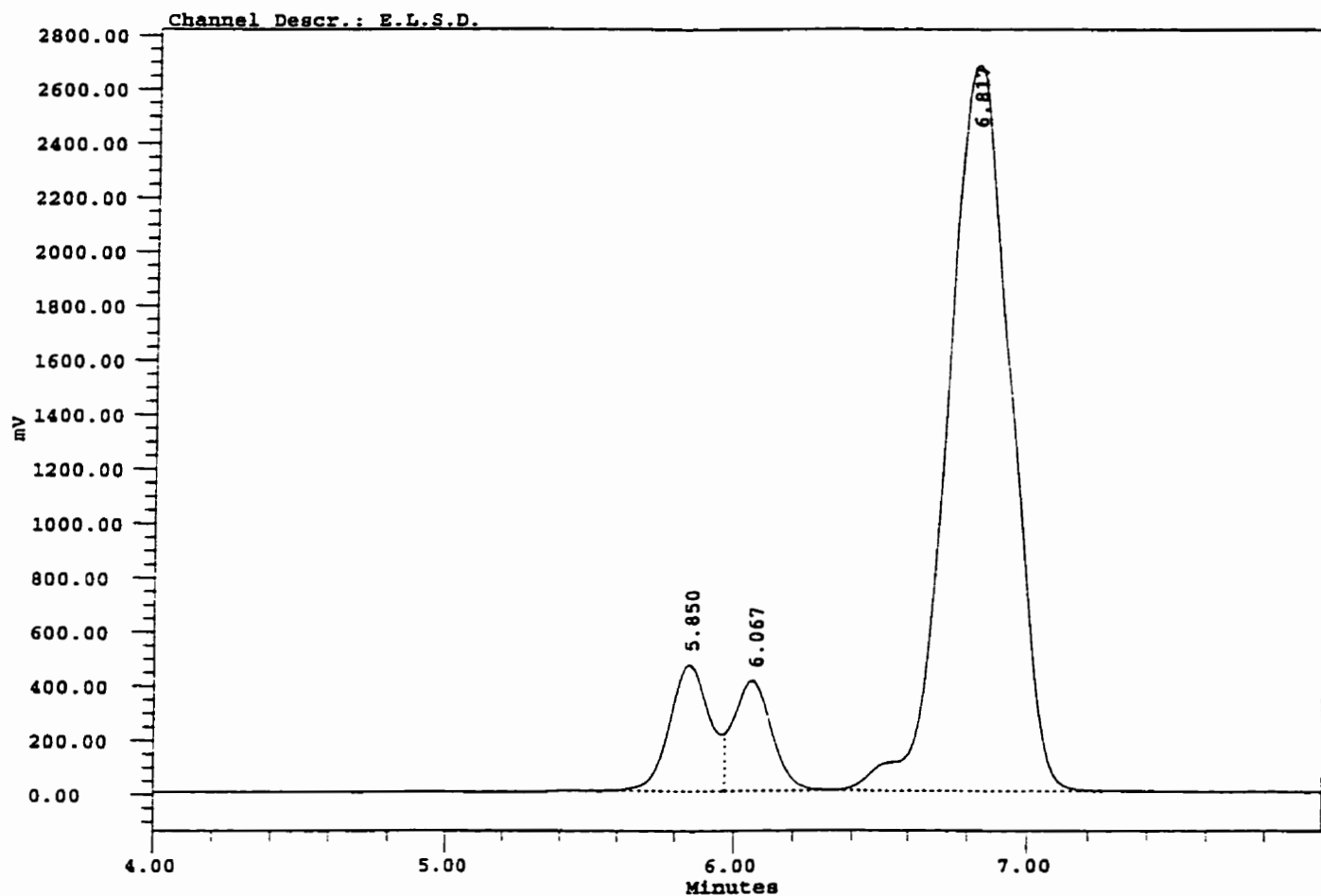
#	Ret Time (min)	Area (μ V*sec)	Height (μ V)	% Area	% Ht	Int Type	Amount	Name
1	5.900	2359440	282974	100.00	100.00	BB		Tristearin
2	6.050					Missing		Diolein
3	6.450					Missing		Monostearin
4	6.617					Missing		Squalene
5	6.700					Missing		Stearic Acid

IV. Tri, di, monoglyceride and free fatty acids of pure beef tallow.

ALLIANCE SAMPLE INFORMATION

Sample Name: #59 Tallow
 Vial: 83 Injection: 1
 Injection Volume: 5.00 uL
 Date Acquired: 04/18/97 12:50:43 AM
 Project Name: Martin_Reaney

SampleInfo: Martin
 Run Time: 8.0 min
 Date Processed 04/18/97 12:59:29 AM
 Processing Method: GPC
 Acq Meth Set: GPC_Styrigel



Peak Results

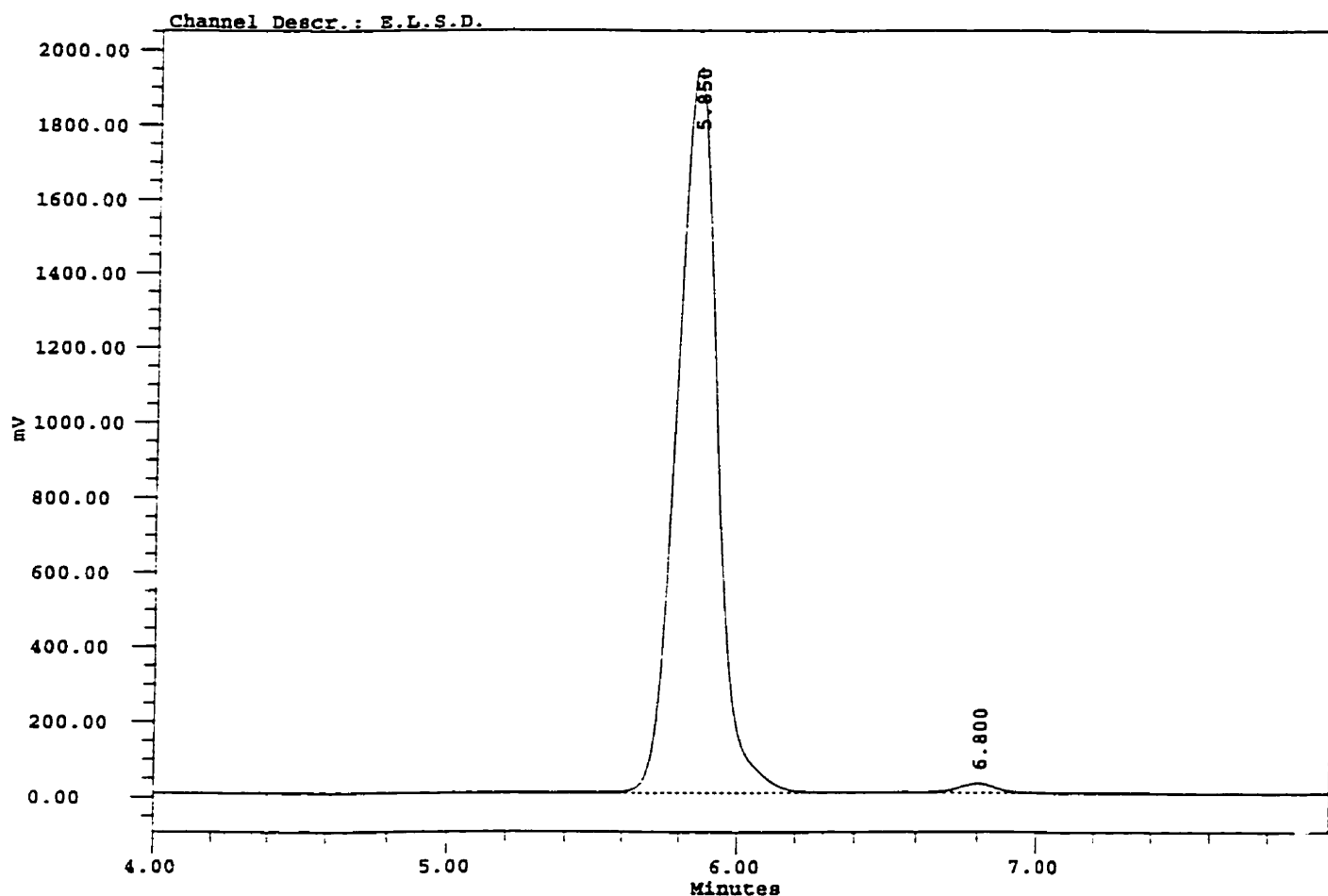
#	Ret Time (min)	Area (uV*sec)	Height (uV)	% Area	% Ht	Int Type	Amount	Name
1	5.850	4463806	465882	9.45	13.14	BV		Tristearin
2	6.067	3622759	403368	7.67	11.11			Diolein
3	6.450					Missing		Monostearin
4	6.617					Missing		Squalene
5	6.817	39136114	2675138	82.88	75.48	VB		Stearic Acid

V. Broiler diet containing 8% tallow and 400 U/g of diet *Pseudomonas* sp. lipase.

ALLIANCE SAMPLE INFORMATION

Sample Name: #55 Tallow
 Vial: 79 Injection: 1
 Injection Volume: 2.00 uL
 Date Acquired: 04/18/97 09:21:35 AM
 Project Name: Martin_Reaney

SampleInfo: Martin
 Run Time: 8.0 min
 Date Processed 04/18/97 09:30:00 AM
 Processing Method: GPC
 Acq Meth Set: GPC_Styrigel

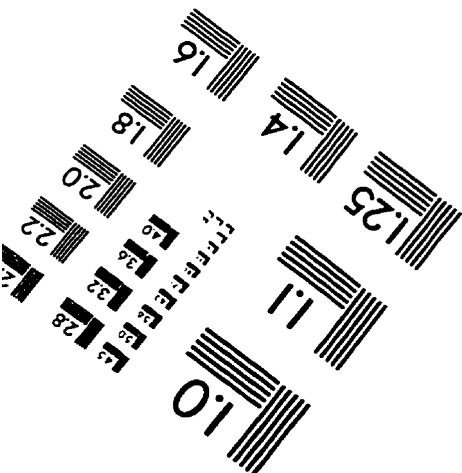
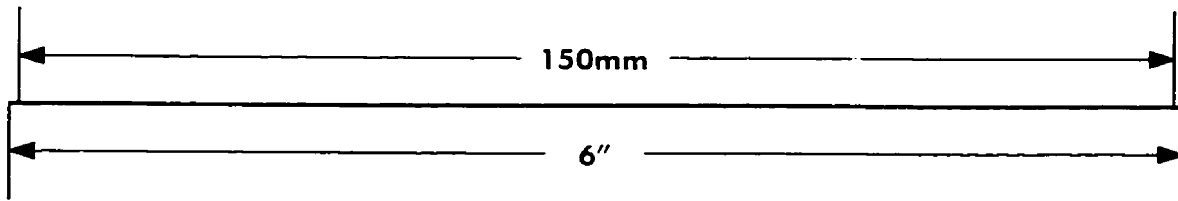
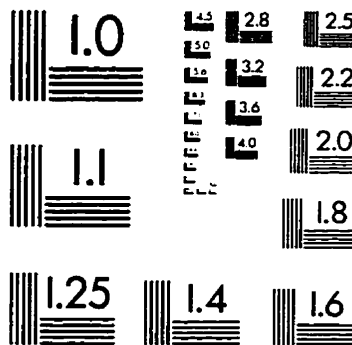
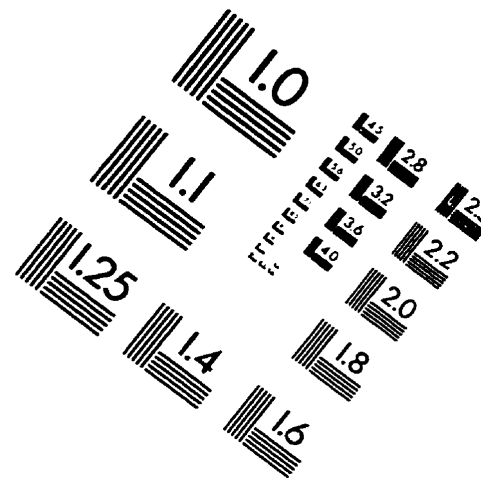
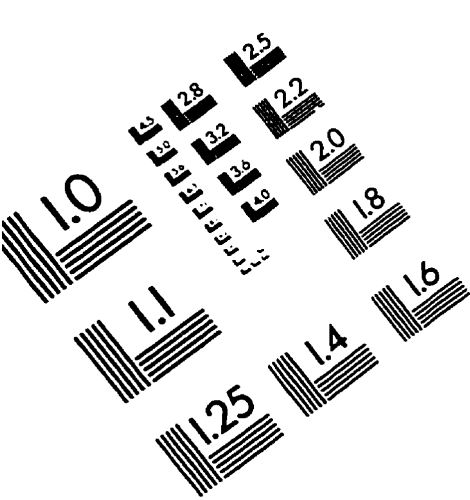


Peak Results

#	Ret Time (min)	Area (uV*sec)	Height (uV)	% Area	% Ht	Int Type	Amount	Name
1	5.850	18567909	1943726	99.94	98.78	BB		Tristearin
2	5.950					Missing		Diolein
3	5.450					Missing		Monostearin
4	5.817					Missing		Squalene
5	6.800	197980	24045	1.05	1.22	BB		Stearic Acid

VI. Broiler diet containing 8% tallow and no added *Pseudomonas* sp. lipase.

IMAGE EVALUATION TEST TARGET (QA-3)



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